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Clinical and Experimental

The Early Disappearance of I^{131} Serum Albumin From the Circulation of Edematous Subjects and Its Implications in the Clinical Determination of the Blood Volume. T. W. Moir, M.D., W. H. Pritchard, M.D., and A. B. Ford, M.D., Cleveland, Ohio 503

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CLINICAL AND EXPERIMENTAL

THE EARLY DISAPPEARANCE OF I^{131} SERUM ALBUMIN FROM THE CIRCULATION OF EDEMATOUS SUBJECTS AND ITS IMPLICATIONS IN THE CLINICAL DETERMINATION OF THE BLOOD VOLUME

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IN A previous communication, a method utilizing the continuous flow of arterial blood past a scintillation crystal, through a small, external A-V fistula, was described which has proved highly practical in following the disappearance of the radioactively-tagged albumin from the circulation in normal subjects.[1] It was shown that the slope of disappearance after mixing was a single exponential function of time and that there was no apparent, inordinate loss of the injected tagged albumin during the early period of recirculation studied.

Studies of the blood volume by use of radioactive red cell tag techniques have shown somewhat smaller volumes than that obtained with simultaneous determinations using dye or radioactively-tagged albumin.[2, 3] The main reason advanced for the apparent larger estimate of the blood volume by albumin tag methods has been that the albumin method measures a space larger than the actual intravascular space by virtue of a presumed, excessive, and unknown diffusion of the tagged albumin from the circulating intravascular space.[4] Since these discrepancies have been shown to exist primarily in patients with edema and heart failure,[5, 6] it became of interest to us to study the disappearance slopes in edematous patients by the recirculation technique.

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The purpose of the present study was: (1) to record the rate of disappearance of labeled albumin from the circulation in edematous subjects and to compare these slopes with the previously obtained normal curves recently reported by us; (2) to examine the time of complete mixing in these edematous subjects; (3) and to calculate and compare blood volumes based on extrapolation of the total curve in these patients with edema from various causes with normals studied by the same technique.[1]

MATERIALS AND METHODS

Technique and methods were identical to those employed in the study of normal subjects.[1] A 4 ml. dilution of approximately 100 μc of iodinated human serum albumin was injected into an arm vein and was followed by a rapid flush of 10 ml. saline. Primary dilution curves were recorded on each individual and during its inscription the arterial needle was allowed to bleed freely into a collecting beaker. Immediately after the cardiac output curve had been recorded, the tubing was transferred from the beaker and fitted into an indwelling venous needle. Continuous flow between the arterial and venous needles was thus established and allowed to proceed during the one- or two-hour study period. Flow in this circuit was approximately 25 ml. per minute.

A single venous sample from another vein on the opposite arm was withdrawn at 10 to 15 minutes after injection of the tagged albumin, and whole blood activity was determined in a well counter for comparison with concentration values obtained by extrapolation of the arterial curve obtained over the entire study period.

Urine was collected at the end of each study period for assay of excreted radioactivity expressed as per cent of the amount injected. In those cases with albuminuria, the urine was fractionated into the aqueous and precipitable protein components and assay of each fraction was done. In one case, radioactivity assay of ascitic fluid was obtained at the end of the study period.

PATIENTS

Ten patients with severe, generalized edema, were selected for study. Of these ten subjects, six had edema secondary to cardiac failure, three had edema of renal origin, and one patient had Laennec's cirrhosis with generalized edema and ascites. All were studied in the morning in the basal state and had received potassium iodide prior to the study period. There were no recognizable hemodynamic changes incident to the creation of the small external A-V fistula.

RESULTS

Fig. 1 shows a typical radioactive output curve with the subsequent disappearance slope obtained in a subject with nephrotic syndrome in whom the study was carried out for a period of 90 minutes. When the disappearance curve, exclusive of the cardiac output curve, is replotted semilogarithmically, the disappearance slope is seen to be a single straight line, exponential function of time (Fig. 2). In Fig. 3 is shown a disappearance curve, replotted semilogarithmically after the cardiac output curve, of one of the cardiac subjects. This exponential disappearance, after the initial period of presumed mixing, was present in all cases studied and is similar to the disappearance curve found in the previous study of nonedematous patients.[1]

In the determination of loss from the circulation following the initial mixing period, the slopes were extrapolated to zero time and the percentage loss was calculated from the theoretic value found at zero time. Although the per cent loss among the edematous patients with cardiac and renal disease is wide

and variable at one hour, the average loss at ten minutes is 1.2 per cent and 2.2 per cent at twenty minutes. These losses compare favorably with the previous calculations on normal subjects, as can be seen from Fig. 4 and Table I.

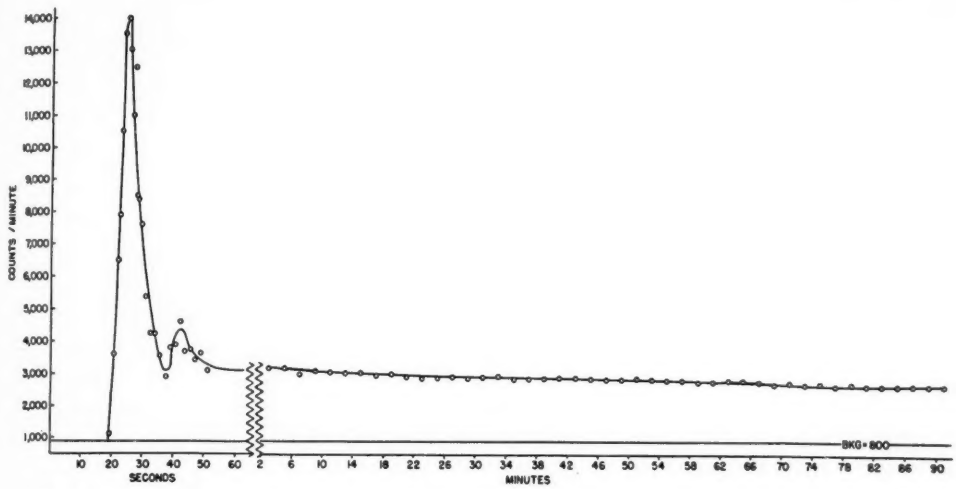

Fig. 1.—Graphic plot of cardiac output curve and disappearance slope in nephrotic patient.

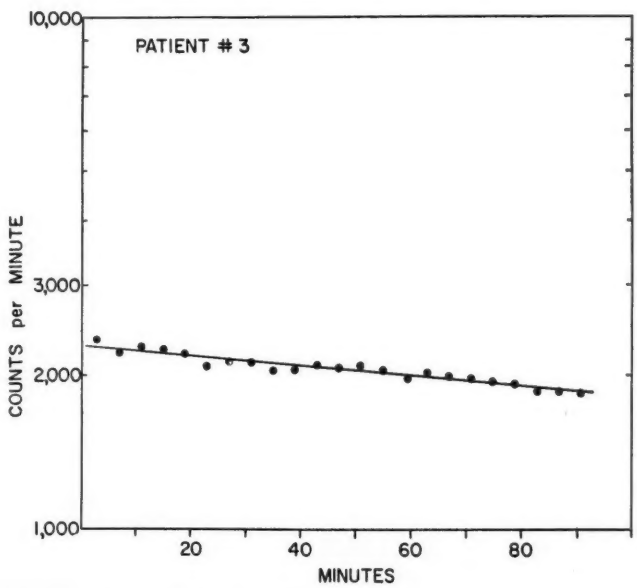

Fig. 2.—Semilogarithmic plot of disappearance slope after inscription of cardiac output curve of Fig. 1.

Thus, as in our normal subjects, these edematous patients, irrespective of the cause of edema, appeared also to exhibit declining concentrations of tagged albumin, which can be expressed as a single exponential function of time up to at least one to two hours, if the initial mixing component be excluded. Losses were variable but not greater than normal during the first 30 minutes.

TABLE I. AVERAGE PER CENT DISAPPEARANCE OF IODINATED (I^{131}) ALBUMIN IN THE NORMAL GROUP AND THOSE WITH EDEMA OF VARIOUS ORIGINS

TIME (MINUTES)	AVERAGE PER CENT DISAPPEARANCE	RANGE (PER CENT)
	NONEDEMATOUS[1]	
10	1.6	1.0- 3.0
20	3.0	1.7- 5.2
60	7.6	3.5-12.8
120	13.5	5.1-23.0
	EDEMATOUS	
10	1.2	0.0 - 2.2
20	2.2	0.58- 4.2
60	7.4	0.58-12.3
120	18.0	15.7 -19.8

TABLE II. DIAGNOSES, MIXING TIME, CARDIAC OUTPUT AND BLOOD VOLUME IN PATIENTS WITH EDEMA

PATIENT	DIAGNOSIS	MIXING TIME (MIN.)	CARDIAC OUTPUT (L./MIN.)	EXTRAPOLATED BLOOD VOLUME (ML.)
1	Cor pulmonale	22	3.02	5,040
2	Rheumatic heart disease	15	5.45	5,730
3	Renal disease	10	2.26	3,144
4	Rheumatic heart disease	10	–	5,625
5	Laennec's cirrhosis	5	5.5	4,160
6	Arteriosclerotic heart disease	16	3.38	5,910
7	Arteriosclerotic heart disease	16	3.38	5,910
8	Renal disease	18	6.45	4,080
9	Cor pulmonale	10	3.77	4,190
10	Renal disease	20	2.32	3,840
Average		14	3.86	4,727

TABLE III. COMPARISON OF BLOOD VOLUMES OBTAINED BY EXTRAPOLATION WITH SINGLE VENOUS SAMPLE IN EDEMATOUS PATIENTS

PATIENT	BLOOD VOLUME		DIFF.	PER CENT DIFF.
	EXTRAPOLATED VALUE (ML.)	10-15 MIN. VALUE (ML.)		
1	5,040	5,050	+10	0.2
2	5,730	5,450	–280	4.9
3	3,144	3,280	+136	4.3
4	5,625	5,250	–375	6.7
5	4,160	4,180	+20	0.5
6	5,910	5,850	–60	1.0
7	5,550	5,490	–60	1.1
8	4,080	4,080	0	0.0
9	4,190	4,190	0	0.0
10	3,840	3,855	+15	0.4
Average	4,727	4,667	–60	1.9

In Table II are shown the values obtained for the time of "complete" mixing, as well as cardiac output, and total blood volume obtained by extrapolation of the disappearance slope. As we have indicated in our previous study we cannot designate a specific point at which "complete" mixing actually occurs, but for our purposes we have designated such mixing as that point where there is no measurable departure from the subsequent extrapolated disappearance slope. The range of mixing times is wide, with a mean value of

14 minutes. This value is 5 minutes longer than the mean mixing time found in the previous normal study by the same method of measurement. Analysis of both sets of data indicates that this difference in the mean mixing time between the edematous and nonedematous subjects is significant, t=2.59,

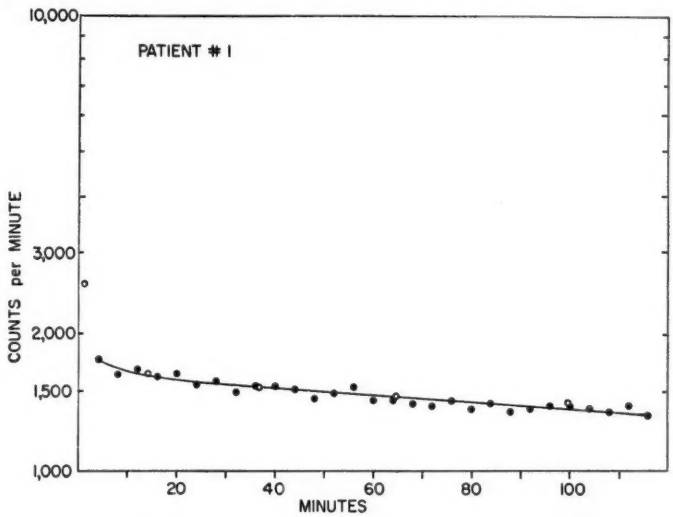

Fig. 3.—Semilogarithmic plot of disappearance slope in typical cardiac showing exponential decline after the period of mixing (22 minutes). Open circles represent venous sample counts.

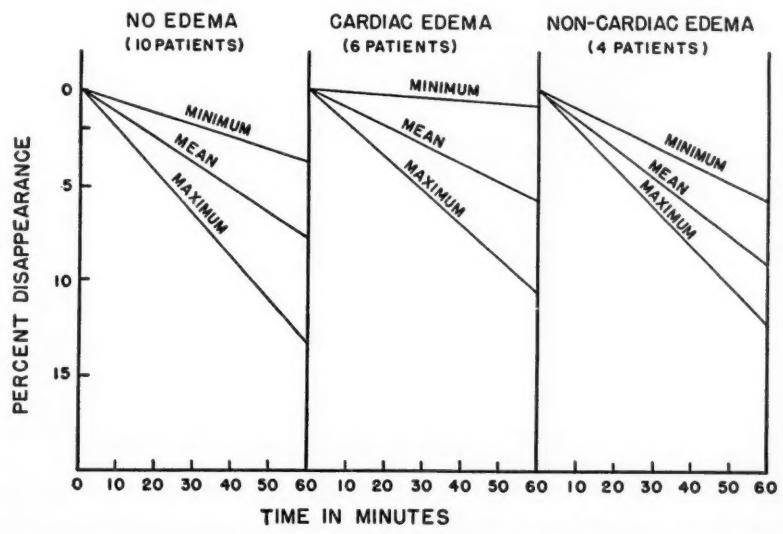

Fig. 4.—Graphic representation of per cent disappearance in normal and edematous subjects during the first hour of study.

$p=>0.01 <0.02$. The cardiac output in these patients was, on the average, low. Furthermore, there was no significant correlation of duration of time of mixing with the cardiac output or the total blood volume.

Table III shows the comparison of the total blood volume obtained by a single venous blood volume sample drawn 10 to 15 minutes after injection of the tagged albumin and that obtained by the extrapolation of the slope of disappearance of the entire study period. It is of interest that the differences are, on the average, surprisingly small in this group of edematous subjects.

TABLE IV. COMPARISON OF BLOOD VOLUME, TOTAL, AND RELATED TO BODY WEIGHT, IN THE TWO GROUPS OF EDEMATOUS SUBJECTS

PATIENT	EXTRAPOLATED BLOOD VOLUME (ML.)	BLOOD VOLUME ML./KG. (WET)
CARDIAC EDEMA		
1	5,040	88
2	5,730	93
4	5,625	80
6	5,910	85
7	5,550	72
9	4,190	88
Average	5,340	84.3
NONCARDIAC EDEMA		
3	3,144	43
5	4,160	39
8	4,080	55
10	3,840	50
Average	3,810	47

TABLE V. COMPARISON OF BLOOD VOLUME, RELATED TO WEIGHT, BETWEEN PREVIOUSLY STUDIED NONEDEMATOUS, AND PRESENT CARDIAC SUBJECTS

NORMALS[1]		CARDIACS	
PATIENT	EXTRAPOLATED BLOOD VOLUME (ML./KG [DRY])	PATIENT	EXTRAPOLATED BLOOD VOLUME (ML./KG [WET])
1	71.5	1	88
5	77.0	2	93
7	53.8	4	80
6	63.0	6	85
12	77.0	7	72
11	65.0	9	88
3	90.0		
9	68.5		
10	77.5		
Average	71.5	Average	84.3

It is apparent from Table IV that there was a definite difference in the total blood volume and in blood volume expressed as milliliters per kilogram of body weight between the cardiac group of patients and the noncardiac edematous subjects. The "wet" weight of these patients was used since any correction factor to establish the "dry" weight is necessarily arbitrary. There is an average difference of 1,530 ml. in total blood volume and 37 ml. per kilogram between the two groups. These differences are highly significant, $t=7.59$, $p=<0.001$. It was of interest also to compare the blood volume of the cardiacs with the normal group obtained by exactly the same technique (Table V). With use of extrapolated curves to calculate blood volume and

with relation of it to the "dry" weight in the normals and "wet" weight in the cardiacs, the difference was 13 ml. per kilogram. This difference is statistically significant, $t=2.59$, $p=>0.02 <0.05$.

The average loss of the radioactivity in the urine two hours after injection in these patients was 2.1 per cent with a range from 0.8 to 6.3 per cent. This average urinary loss was somewhat higher in the patients with noncardiac edema (2.63 per cent) than in the cardiacs (1.4 per cent). The latter figure is within the range of the urinary losses of radioactivity found at the end of two hours in our normals but is slightly higher than the 0.87 per cent figure found by Schultz and associates.[7] The higher urinary activity present in the nephrotic patients is due to the presence of marked albuminuria with subsequent loss of the still tagged albumin molecule. This was verified by the finding that the majority of the excreted radioactivity of these patients was present in the precipitable protein fraction of the urine when this was separated and washed.

In the patient with cirrhosis, ascitic fluid obtained two hours after injection of the tracer dose of the I^{131}, serum albumin contained only 0.67 per cent of the injected radioactivity.

DISCUSSION

Recent reports of low or normal blood volume in cardiac failure as measured by radioactive red cell tags have challenged the classic concept of hypervolemia as an inherent defect in the hemodynamics of cardiac insufficiency. Since the latter concept grew out of blood volume determinations obtained by labeled albumin,[8-10] there has been much debate recently regarding the accuracy of such tags for estimating blood volume in the presence of edema and cardiac failure. There are some who are opposed to the use of albumin tags in principle, since the molecule is known to diffuse from the intravascular space.[4] However, few attempts to quantitate these losses are on record. Thoracic duct studies in animals[11] and in man[7] have shown losses of variable quantities of injected labeled albumin, with maximum loss progressive after 10 to 20 minutes following injection and with negligible amounts of albumin appearing in the thoracic duct prior to ten minutes. However, it is to be noted that these anatomic approaches in reality measured the loss at the time of return of the labeled albumin to the venous circulation.

Our study indicates that after a variable period of "mixing," slopes of disappearance in edematous subjects were no steeper than in a group of normal patients similarly studied. This observation does not bear out the arguments of those who state that the loss of albumin is accelerated in the presence of edema, particularly that of cardiac origin.[4] It should be emphasized, however, that the losses of injected albumin calculated in the present studies have been based on the rate of fall of the slope occurring after: (1) the initial primary dilution curve of the first circulation and (2) the immediate rapid decline prior to the onset of the subsequent exponential disappearance. Losses which may occur during these two periods are not detectable by the present method of study. In normal patients, this loss must be small, indeed, since cardiac

outputs based on the primary dilution curve and a ten-minute sample for estimating blood volume have agreed well when compared with other output methods.[12] However, in the presence of edema, the possibility that the losses during this initial period after injection may be large must be considered.

In the classical concept of American workers, the early, more rapid decline of concentration after injection and before the later exponential fall occurs, has been considered to be due to mixing. More recent evidence for this is the failure of in vivo counting over the peripheral tissues to reach a plateau during this first ten-minute period.[13] Some European workers, however, have extrapolated this early "mixing" period and have assumed the concentration at zero time to represent the dilution by the true intravascular volume. The lower volume obtained by this method more nearly approaches that obtained by the red cell tag methods for prediction of blood volume.

To determine possible increased losses during this initial period in the presence of edema, we have also extrapolated this mixing slope in both the present series and in the former study of nonedematous subjects. In the normal subjects, the per cent increase in concentration over the extrapolated values obtained from the disappearance slope averaged 9.2 per cent (range 6 to 12 per cent). Among the edematous subjects the increase in concentration by extrapolation of this "mixing" slope average 8.2 per cent (range 9 to 16.4 per cent). When those patients with cardiac edema were examined separately the average increase in concentration by this method was 11.0 per cent with a range from 6.9 to 16.4 per cent. With use of these mixing slope concentrations, the blood volume calculations became 10 per cent lower in normals, in the patients with cardiac edema, and in those patients with edema of noncardiac origin. Thus, even if one uses the disappearance slope during this early period for the calculation of the blood volume, there is no difference between normal patients and those with edema; and although on the average, one finds the estimated blood volume some 10 per cent lower than by the usual extrapolation techniques, this is considerably less than the differences of 25 per cent found by red cell tag methods when compared to albumin tags in edematous subjects.[6]

Although extrapolation of the early mixing phase gives smaller blood volume values in general, the magnitude of the discrepancies often found in cardiac failure are not explained; nor can one justify the use of a slope of a period during which injected material has not been distributed throughout the vascular bed. To date there are no data from which to distinguish the falling concentration due to intravascular mixing from that due to diffusion from the intravascular space of the tagged albumin. We have demonstrated, however, that there appears to be one rate of exponential diffusion once this initial period has been passed and that the slopes defining this diffusion are no steeper in edematous subjects than in normal patients. Experiments designed to determine whether a normal, small, but rapidly equilibrating extravascular space exists and expands greatly in cardiac failure are in progress.

As suggested by the proponents of the red cell tag methods, inadequate mixing of the injected tag before sampling could lead to an erroneously low

estimate of the blood volume in cardiac failure.[5] Our estimate of albumin mixing in the present group of edematous subjects indicates that the mixing time is prolonged over that found in the normal subjects. It is evident that premature sampling is an inherent error, leading to an erroneous low estimate of blood volume, in any state of slow mixing of which cardiac failure is one. Altered distribution of the red cell tag after injection and differences between venous and whole body hematocrit may be other sources of discrepancy.

The finding of essentially similar total blood volumes as estimated by the single 10- to 15-minute sample and that obtained by extrapolation of the slope of disappearance over the entire study was somewhat surprising in the present study. With the delayed mixing time evident in these edematous subjects, one would have expected the 10- to 15-minute blood volume to be uniformly lower than the extrapolated values. This finding is probably fortuitous in the sense that the single, early sample obtained during the mixing period "compensates" for variable losses after the mixing had been complete. Estimates of the blood volume in these abnormal states are more reliably obtained by the extrapolation technique if serial sampling is begun after the period of mixing. The present study further suggests that sampling for extrapolated blood volumes in cardiac failure should not begin prior to 15 minutes after injection of the tracer substance.

SUMMARY AND CONCLUSIONS

1. The disappearance rate of I^{131} serum albumin from the circulation of 10 subjects with severe, generalized edema has been studied by a recirculation technique utilizing continuous arterial flow of blood past a scintillation crystal. Regardless of the etiology of edema, the disappearance slope was exponential after mixing up to at least one hour. Loss of iodinated (I^{131}) albumin, expressed as percentage of the initial theoretical concentration at zero, appeared to be no greater than in a group of normals similarly studied previously.

2. The average mixing time of this group of patients was longer than the group of patients without edema.

3. Blood volume was significantly elevated in subjects with edema of cardiac origin and significantly reduced in those whose edema was of nephrotic or cirrhotic origin.

4. In the employment of albumin-tagged substances for blood volume determinations in patients with edema, samples drawn after thirty minutes and up to one to two hours should give a single exponential slope from which accurate calculations can be made. The practical expedient of drawing four samples at 10-minute intervals from the 30 to 60 minutes following injection is recommended.

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PERIPHERAL VENOUS OXYGEN SATURATION IN PATIENTS WITH AND WITHOUT LIVER DISEASE

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IN THE course of routine antecubital venipunctures in several patients in hepatic coma and severe liver decompensation, it was noted that venous blood appeared arterial in color. The existence of arteriovenous anastomoses in the hand is well known.[2] A palmar erythema occurs in liver cirrhosis and is characteristically localized to areas where arteriovenous anastomoses are numerous.[6] In cirrhosis, Abelmann and associates[7] found resting cardiac output high and oxygen consumption proportionally small. In explanation they suggest arteriovenous shunting of blood in the skin. Data on peripheral venous oxygen saturation in patients with liver disease were not found in the literature reviewed. Therefore some observations were made to determine whether the peripheral venous oxygen saturation is higher in patients with liver disease than in controls.

MATERIAL AND METHODS

Five patients with obvious liver disease were studied (Cases 1 to 4, cirrhosis; Case 5, hepatitis). Control studies were made in four cases with no evidence of liver disease. In Patient 1 there was bleeding from esophageal varices, and the patient died in hepatic coma. Patient 2 had a primary hepatoma in a cirrhotic liver and died after ten days of coma. Post-mortem examinations were performed in Cases 1 and 2. Patient 3 was precomatose but recovered. Patients 4 and 5 were alert. One of the four control patients had cutaneous blastomycosis and was under treatment with 2-hydroxystilbamidine. There was no evidence of hepatic injury. One patient had finished his course of antibiotic therapy with penicillin and streptomycin for bacterial endocarditis and was ambulatory and afebrile. The other two were cases of gastric carcinoma and acne vulgaris.

All patients were supine and at complete rest for at least one hour prior to drawing samples. Blood was drawn without stasis from the femoral vein and artery and antecubital vein. Except in Patients 1 and 2, the needle was left in place in the antecubital vein, and a second sample was withdrawn after flow from the hand vessels was interrupted. This was done by applying a pressure of 200 mm. of mercury at the wrist with a sphygmomanometer cuff. All blood samples were drawn anaerobically and analyzed for oxygen content.[1] The methods of determining the oxygen saturation are indicated in Table I. In some cases carbon dioxide content[1] and pH[9] were also determined. Room temperature (except in Cases 1 and 2), rectal body temperature, and gross skin temperature were recorded at the time of drawing blood.

Room temperature was comparable for the two groups. Rectal temperatures were normal in all patients studied. Blood pressure and pulse were normal and similar in the two groups, except for two episodes of moderate hypotension in Patient 1 (second determination) and Patient 2 (third determination).

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TABLE I. OXYGEN CONTENT, SATURATION, AND ARTERIOVENOUS DIFFERENCE OF BLOOD FROM ANTECUBITAL AND FEMORAL VEINS AND FEMORAL ARTERY

PATIENT	AGE, SEX	DIAGNOSIS	ROOM TEMP. (°C.)	ANTECUBITAL VEIN (ACV) NO CUFF CONTENT VOL. %	ANTECUBITAL VEIN (ACV) NO CUFF SATURATION %	ANTECUBITAL VEIN (ACV) WRIST CUFF CONTENT VOL. %	ANTECUBITAL VEIN (ACV) WRIST CUFF SATURATION %	FEMORAL VEIN (FV) CONTENT VOL. %	FEMORAL VEIN (FV) SATURATION %	FEMORAL ARTERY (FA) CONTENT VOL. %	FEMORAL ARTERY (FA) SATURATION %	A-V DIFFERENCE FA-ACV CONTENT VOL. %	A-V DIFFERENCE FA-FV CONTENT VOL. %
							Liver Disease						
1. D. R.	18, M	Cirrhosis	24.0*	10.6	80.2					12.1	91.5	1.6	
			24.0*					9.6	74.4				
			24.0*	15.5	83.8					16.5	89.1	1.0	
			24.0*	16.1	87.5					16.3	88.2	0.2	
2. V. L.	62, M	Cirrhosis	24.0*	14.0	92.5					14.3	94.3	0.3	
			24.0*	14.2†	86.5			10.9	66.0	15.6	93.8	1.4	4.7
			24.0*	12.2	88.3			8.5	62.2	12.5	90.6	0.3	4.0
3. C. J.	58, F	Cirrhosis	24.0*	14.8	91.1			11.7	72.5	16.0	98.0	1.2	5.0
			25.2	14.7	90.7	10.9	62.5	8.4	53.6	14.7	90.7	0.0	6.3
4. K. B.	43, F	Cirrhosis	23.0	17.9	84.8	13.9	68.4	14.9	69.6				
5. L. T.	33, F	Hepatitis	26.8	13.9	90.2	12.0	74.5						
Average	43		24.5		87.7		68.5		67.8			0.7	5.0
							No Liver Disease						
6. W. H.	41, M	Blasto-mycosis	24.1	17.9	86.0	15.1	72.8	11.1	52.6	19.5	90.2	1.7	8.4
7. M. A.	30, M	S.B.E.	23.5	10.4	59.5	9.2	52.6	13.6	80.2	16.8	92.3	6.4	3.2
8. O. C.	71, M	Gastric Carcinoma	24.5	8.7	46.0	9.7	51.3	11.1	58.8	18.0	95.0	9.3	7.9
9. J. B.	27, M	Acne	24.5	15.4	78.9	15.5	79.4	15.0	77.3	18.4	95.0	3.0	3.4
Average	42		24.2		67.6		64.0		67.2			5.1	5.7

Oxygen saturation $= \frac{\text{content}}{\text{capacity}} \times 100$. Capacities were obtained by (1) direct measurements in Patients 1 and 2 and in the first determination of Patient 3, and (2) calculation—(a) from the hemoglobin in Patients 3-7 (Hb × 1.34 vol. %/Gm.) (b) from the assumption that the arterial sample was 95% saturated in Patients 8 and 9.

*Approximate.

†Slight stasis.

RESULTS

These are summarized in Table I. The average oxygen saturation of antecubital venous blood in the five patients with liver disease was 87.7 per cent (range—80.2 to 92.5—10 determinations). In the four patients without hepatic disease the average value was 67.6 per cent (range—46.0 to 86.0—4 determinations). When the hand circulation was occluded by pressure, the average antecubital venous oxygen saturation fell to 68.5 per cent in three patients with liver disease and to 64.0 per cent in controls. The average femoral venous oxygen saturation was similar in the patients with hepatic disease and in the controls (hepatic disease—67.8 per cent, controls—67.2 per cent). The average arteriovenous oxygen difference for the antecubital vein (hand circulation not interrupted) was 0.7 vol. per cent in three cases of liver disease (8 determinations). In four controls it was 5.1 vol. per cent.

Blood CO_2 and pH were determined in conjunction with oxygen in some cases. In four determinations on two patients with severe liver disease, the average A-V CO_2 difference for the antecubital vein was 1.71 vol. per cent, while in two control patients it was 6.6 vol. per cent on two determinations. The average A-V difference for the femoral vein was 4.1 vol. per cent in two cases of severe liver disease, while 6.1 vol. per cent was obtained in two control cases. The average A-V pH difference for the antecubital vein in five determinations on two hepatic patients was 0.023, while in three determinations in patients with liver disease an A-V difference of 0.064 was obtained for the femoral vein.

DISCUSSION

The average resting antecubital venous oxygen saturation of 67.6 per cent and the wide range (46.0 to 86.0) in the control patients agrees well with the data of Keys,[8] who found an average antecubital venous oxygen saturation of 68.2 per cent, with a range of 25 to 85 per cent, in 63 normal individuals. On the other hand, the average antecubital venous oxygen saturation of 87.7 per cent, with a range of 80.2 to 92.5 per cent, in patients with liver disease in the present study is decidedly higher than Keys's value for normals. The average femoral venous oxygen saturations (67.8 per cent in cases of liver disease and 67.2 per cent in controls) is similar to the value of 71.2 per cent given by Keys for normal individuals. He also found that differences in serial determinations in the subjects accounted for a large share of the total group variation of oxygen saturation of antecubital venous blood. In the present study, multiple determinations in three patients with liver disease (Cases 1, 2, and 3) yielded consistent values in each case.

The approximation of antecubital venous oxygen saturation to that of femoral venous blood when the hand circulation is completely interrupted localizes the differences between the two limbs to the hand circulation. Blood flow in the hand may increase more than a hundredfold, with large increases in ambient temperature.[10] The hand has the highest concentration of arteriovenous anastomoses in the body,[2] which are largely responsible for the great capacity for variation in hand blood flow and for the oxygen saturation in hand

veins being higher than that in the antecubital veins.[11] Hand-warming results in high blood flow[4] and high antecubital venous oxygen saturation,[3] due to the opening of arteriovenous anastomoses.[12]

Arteriovenous shunting of blood in the hands would account for the high oxygen saturation in the cases with liver disease. In support of the thesis that there is a greater amount of arteriovenous shunting in the hand in liver disease than in control patients, Abramson and Lichtman[5] found that the average hand blood flow of six patients with liver disease was twice that of six normal patients. Of the five patients with liver disease in the present study, warm hands were noted in all; palmar erythema existed in four. Spider nevi were absent from the two fatal cases (Patients 1 and 2).

An increased amount of arteriovenous shunting in liver disease could conceivably be due to an increased absolute number of arteriovenous anastomoses or to an increased number of functioning units. The latter, in turn, might be due to an abnormal substance released by the liver or to an increased amount of a substance normally removed or altered by the liver, for example, estrogen.

Reflex dilatation of blood vessels of the hands would account for an increase of only 250 ml. per minute in cardiac output.[13] It is likely that A-V shunting occurs at other sites in addition to the hand, since Abelmann and associates[7] found larger increases in cardiac output than 250 ml. in patients with cirrhosis as compared with normals. As warmth of the skin in general was often noted in liver disease, there may actually be a relative vasodilatation in the skin of the body in general to account for at least a part of the increased cardiac output. Another factor of possible importance here is increased arteriovenous shunting in the lung. Rydell and Hoffbauer,[14] in this laboratory, have recently studied a case of cirrhosis in which cyanosis and clubbing were shown to be due to many small A-V communications in the lung.

The warmth of the feet, as well as the hands, in the present series of liver disease, and the known presence of arteriovenous anastomoses in the toes and part of the plantar surface of the feet, makes it likely that A-V shunting occurs in the foot in liver disease. This may have been masked by the greater proportion of muscle in the lower extremity than in the hand and forearm.

Arteriovenous anastomoses in animals have been demonstrated by various techniques for many organs.[15] In man, A-V anastomoses have been found consistently in the penis, hands and feet, and glomus coccygeum,[2] while there is recent evidence of their presence in the stomach.[16] Bradley and associates[17] found decreased blood flow and increased oxygen A-V difference for the liver in cirrhosis as compared to normals. Therefore, it would appear that blood flow through the liver, exiting in hepatic veins, does not contribute to the increased cardiac output and diminished cardiac arteriovenous oxygen difference noted by Abelmann and co-workers[7] in liver cirrhosis. It is conceivable, however, that a large portal venous blood flow through anastomotic extrahepatic vessels, with its relatively high oxygen content under resting conditions,[19] could contribute to the increased cardiac output and diminished A-V oxygen difference.

Abnormal anastomotic vessels have been described in the cirrhotic liver. Popper and associates[18] have found "relatively few anastomoses between branches of the portal vein, more between branches of the portal vein and hepatic artery, and many" . . . small calibered anastomoses . . . "between branches of the portal and hepatic veins." The latter would appear to be a by-pass of the liver parenchyma by portal blood, an internal Eck-fistula, to which the authors attach great significance in the pathogenesis of cirrhosis of the liver.

Clubbing, which is said to occur with increased blood flow,[6] was found in only one of the present cases of liver disease, while one patient without liver disease, with subacute bacterial endocarditis, had clubbing with an antecubital venous oxygen saturation of 49.5 per cent and an arterial saturation of 92.3 per cent. The lack of correlation of high antecubital venous oxygen saturation and presence of vascular spiders is not surprising, since Bean[6] emphasizes that the vascular spider is not an arteriovenous anastomosis since it empties into a capillary bed. It is interesting, however, that the arterial portion of vascular spiders is coiled and contains glomus cells in its wall, as do arteriovenous anastomoses.[6, 2]

The finding of a marked cardiac systolic murmur in the patient with precoma and clubbing and one of lesser intensity in the patient in coma, with no evidence of valvular lesions at autopsy, may be due to increased cardiac output and blood turbulence, analogous to the murmur found in severe anemia.

SUMMARY AND CONCLUSIONS

1. The average arteriovenous oxygen difference for antecubital venous blood was smaller and the oxygen saturation higher in five patients with liver disease than in four controls. There was some overlapping between the two groups.

2. Arteriovenous differences in CO_2 and pH appeared to follow the same trend as the oxygen determinations, insofar as the limited data permit any conclusion.

3. An increase in arteriovenous shunting in the hands in patients with liver disease is believed to account for the differences observed.

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THE "CORRECTED" BROMSULFALEIN TEST

A Comparison With the Cephalin-Cholesterol Flocculation Test and the Thymol Turbidity Reaction in Jaundiced Patients

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THE measurement of bromsulfalein retention as a test of hepatic function, introduced by Rosenthal and White in 1925,[1] has gained wide acceptance. It has generally been assumed that the test is not reliable in the presence of jaundice. Characteristic opinions such as the following are common in the current literature: "the bromsulfalein test has practically no value in the presence of jaundice" (Magath[2]); "it is the prevailing opinion that its greatest value (the B.S.P. test) is in the diagnosis of liver damage in patients without hyperbilirubinemia" (Lichtman[3]); "this test is of clinical value in cases of hepatic damage in which no jaundice is present" (Weiss[4]). The contraindication to the performance of the test in the presence of jaundice is not primarily due to technical difficulties, since Geabler's[5] method utilizing the photoelectric colorimeter permits the determination in icteric or turbid serum.

In 1951, Zieve, Hanson, and Hill[6] derived a "correction factor" that was said to allow one to calculate the bromsulfalein value that *would have been obtained* had significant jaundice not been present. These investigators reasoned that the bromsulfalein value observed in jaundiced patients consisted of two parts: bromsulfalein retained in the serum and bromsulfalein returned to the serum because of regurgitation. They sought to segregate these two portions and calculated a score for the serum bromsulfalein due to the regurgitation mechanism. Zieve and associates studied the relationship of the level of the one-minute serum bilirubin to the concentration of the dye retained in the blood at 45 minutes. In 37 patients with one-minute serum bilirubin levels greater than one mg. per 100 ml., a significant correlation was said to obtain. From these observations, a "correction factor" was devised by which the observed bromsulfalein value could be reduced to a figure that presumably would have been obtained had not the dye "regurgitated" after preliminary extraction by the liver. For example, a B.S.P. value of 38 per cent obtained in a patient whose one-minute serum bilirubin value is 10 mg. per 100 ml. is reduced to 10 per cent by subtraction of this correction factor.

During the four-year period (1951-1954), bromsulfalein tests were performed on a large number of jaundiced patients at the University of Minnesota

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TABLE I. RESULTS IN CASES OF EXTRAHEPATIC OBSTRUCTION DUE TO CARCINOMA

CASE	SEX	AGE (YEARS)	DURATION OF JAUNDICE (WEEKS)	SERUM BILIRUBIN		B.S.P.		THYMOL TURBIDITY	CEPHALIN CHOLESTEROL (24 HOURS)	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	ACTUAL (%)	CORRECTED (%)			
1	M	28	1	2.3	3.4	42	32	3	1+	Necropsy
2	M	77	12	2.4	3.6	12	3	3	2+	Operation
3	M	66	4	2.7	4.6	30	18	3	3+	Clinical data
4	F	76	10	3.2	5.3	59	45	2	0	Operation
5	F	74	8	5.5	9.0	26	5	3	tr.	Operation
6	M	75	1	7.3	11.4	38	15	1	0	Operation
7	M	70	8	7.9	13.4	66	41	4	2+	Clinical data
8	F	68	8	8.3	11.7	64	38	1	0	Operation
9	F	64	1	8.5	12.5	44	18	7	tr.	Operation
10	F	70	3	8.9	13.8	56	30	4	2+	Operation
11	M	70	3	9.5	13.0	62	35	12	0	Operation
12	F	77	5	10.5	17.2	36	8	6	2+	Necropsy
13	M	77	3	11.0	18.1	48	19	1	0	Necropsy
14	M	66	3	11.5	22.0	36	5	9	0	Operation
15	M	54	1	13.0	23.5	36	5	7	0	Operation
16	F	70	2	13.0	22.0	28	0	2	4+	Operation
17	M	79	4	13.9	22.2	42	10	2	tr.	Necropsy
18	F	63	8	14.0	24.5	48	16	4	0	Operation
19	M	67	6	14.0	21.7	36	4	2	2+	Necropsy
20	M	80	4	14.6	23.7	38	2	3	2+	Necropsy
21	M	57	6	15.5	28.8	32	0	4	2+	Operation
22	M	84	4	16.3	31.1	40	6	1	tr.	Clinical data
23	F	67	6	16.3	33.3	33	0	4	1+	Operation
24	M	56	20	17.5	31.0	42	7	4	4+	Clinical data
25	F	70	8	18.5	34.0	21	0	5	0	Operation
26	M	57	8	21.8	34.7	54	17	3	tr.	Clinical data

Hospitals. The values obtained were "corrected" according to the Zieve-Hanson-Hill formula. The results in 111 icteric patients have been analyzed. The criteria by which one can judge the merits of hepatic tests are limited. One means of evaluating the clinical usefulness of a given test is to ask: Does it assist in distinguishing "medical" from "surgical" types of jaundice; that is, does the test discriminate jaundice due to intrahepatic obstruction from that due to extrahepatic obstruction? The popularity enjoyed by such widely used tests as the thymol turbidity and the cephalin-cholesterol flocculation test is due, in large part, to this particular advantage. In the present study, the behavior pattern of the "corrected" bromsulfalein test in the jaundiced patient has been analyzed with respect to its utility in such a differentiation. The merits of this test have been compared with those of the cephalin-cholesterol and the thymol turbidity tests performed simultaneously.

METHODS

Patients selected for this study include only those in which the one-minute serum bilirubin value (method of Ducci and Watson[7]) exceeded one mg. per 100 ml. The percentage of bromsulfalein dye retained at 45 minutes (5 mg. per kilogram of body weight dosage) was corrected by the Zieve-Hanson-Hill formula. The clinical records of all patients were carefully reviewed and only those with definitely established diagnoses were selected for analysis. Of 111 patients so studied, 42 had jaundice due to extrahepatic obstruction (carcinoma, 26, and common duct stone, 16). The 69 patients with intrahepatic obstruction included 43 patients with cirrhosis and 26 patients ill with viral hepatitis. The means by which the diagnoses were established are shown in Tables I, II, III, and IV. Patients with jaundice due to miscellaneous causes such as multiple liver abscesses and Hodgkin's disease involving the liver were omitted, since it was felt that the various factors producing jaundice could not be distinguished. Eighty-three per cent of the diagnoses of extrahepatic obstruction were confirmed by either autopsy or exploratory laparotomy. The cases with viral hepatitis conformed to the classical picture of this disease; the abnormal liver function studies returned to normal as the patients recovered. In the one fatal case, autopsy was performed. Seventy-five per cent of the cases of jaundice due to cirrhosis were confirmed by autopsy, liver biopsy, or peritoneoscopy. Most of the remainder had been followed for a number of years at the University Hospital and the diagnosis of cirrhosis was well established.

RESULTS

The data available for analysis are presented in Tables I, II, III, and IV. The results in the cases of extrahepatic obstruction (Tables I and II) indicate the unpredictability of the corrected B.S.P. test. If the proposed correction factor is reliable, the bromsulfalein retention value should, presumably, correct to a normal value (5 per cent or less) in cases of *uncomplicated* obstructive jaundice. It is apparent from the values in Table I that such did not occur in the present series of cases with obstruction due to cancer. In the 16 cases of extrahepatic obstruction due to common duct stone, a corrected bromsulfalein value below 5 per cent was observed in only two instances.

The data from 69 cases of intrahepatic obstruction (jaundice due to hepatitis or to cirrhosis) are shown in Tables III and IV. In these patients the

TABLE II. RESULTS IN CASES OF EXTRAHEPATIC OBSTRUCTION DUE TO COMMON DUCT STONE

CASE	SEX	AGE (YEARS)	DURATION OF JAUNDICE	SERUM BILIRUBIN		B.S.P.		THYMOL TURBIDITY	CEPHALIN CHOLESTEROL (24 HOURS)	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	ACTUAL (%)	CORRECTED (%)			
27	M	70	4 years	1.4	2.8	27	23	5	tr.	Operation
28	F	28	2 years	1.5	3.0	23	18	3	3+	Operation
29	M	76	1 year	2.4	4.4	46	35	2	tr.	Clinical data
30	M	68	1 week	2.6	4.2	30	18	3	0	Clinical data
31	F	71	2 years(?)	2.9	5.3	14	1	3	1+	Operation
32	F	69	6 weeks	3.0	6.8	28	15	2	0	Operation
33	M	77	1 week	3.5	5.7	28	13	8	tr.	Operation
34	F	40	32 weeks	4.5	6.5	48	30	4	2+	Operation
35	M	42	2 weeks	4.7	7.4	28	9	12	0	Operation
36	F	40	5 weeks	5.8	7.9	80	60	6	1+	Operation
37	F	60	3 weeks	6.5	11.5	46	23	2	0	Operation
38	F	63	1 week	7.5	12.2	48	24	6	0	Operation
39	F	62	4 weeks	9.0	14.0	54	27	3	0	Operation
40	M	57	1 week	9.3	15.6	44	17	2	0	Operation
41	M	73	2 weeks	11.8	16.2	58	28	3	0	Operation
42	F	69	3 weeks	25.0	42.0	40	0	4	0	Operation

corrected bromsulfalein did conform to the anticipated results. The data from the bromsulfalein tests in the 111 patients are presented in graphic form in Figs. 1 and 2. The actual B.S.P. values obtained are plotted against the one-minute serum bilirubin in Fig. 1; in Fig. 2 the corrected B.S.P. value has been similarly plotted. The degree of bromsulfalein retention in the 111 jaundiced patients did not correlate well with the type of disease or disorder present.

In order that a comparison might be made between the relative merits of two more simple laboratory tests (cephalin-cholesterol flocculation reaction and thymol turbidity) with the corrected B.S.P. test, an arbitrary classification of "informative," "equivocal," or "misleading" was made, as shown in Table V. When the cephalin-cholesterol flocculation test is employed as a laboratory aid in the differential diagnosis of jaundice, a 3-plus or 4-plus reaction may be considered "informative" in a patient with hepatitis or cirrhosis (intrahepatic obstruction). However, a 3-plus or 4-plus reaction is "misleading" in a patient with jaundice due to stone or cancer (extrahepatic obstruction). As indicated in Table VI, the cephalin-cholesterol test was, in this sense, informative in 78 per cent of the patients with intrahepatic obstruction and in 71 per cent of those with extrahepatic obstruction. A similar type of analysis can be applied to the thymol turbidity test where values greater than 7 units may be termed informative in patients whose jaundice is due to intrahepatic disorders (hepatitis or cirrhosis in this series). Thymol turbidity values of 4 units or less are anticipated (though not always observed) in patients with jaundice due to cancer or to stone obstruction. Hence a value of 4 units or less in such patients has been termed informative. In this series of 111 patients, the thymol test was informative in 65 per cent of the cases of intrahepatic disease and in 74 per cent of the patients with extrahepatic obstruction. The limitations of these two laboratory procedures are well known and it is not surprising therefore that the results in any series of cases fall far short of perfection in their ability to predict the nature of the patient's jaundice. As noted (Table VI), the two tests were frankly misleading in 13 per cent of the 69 cases of jaundice due to hepatitis or cirrhosis and in 10 per cent of the 42 cases of jaundice due to stone or cancer. The tests were termed "equivocal" (Tables V and VI) where the values obtained were intermediate.

A similar comparison of the corrected B.S.P. test has been made. The arbitrary limits set up for this test, as indicated in Table V, were liberal. The test was considered informative if the corrected B.S.P. value was 10 per cent or less in the presence of jaundice due to extrahepatic obstruction. A value of 20 per cent or more was demanded if the test was to be regarded as informative in the patient with hepatitis or cirrhosis. As will be noted, the performance of the corrected B.S.P. test was inferior to either the cephalin-cholesterol test or the thymol turbidity when judged by these standards. Its performance was unreliable, as a diagnostic aid, in cases of jaundice due to extrahepatic obstruction. In that group of patients the results were misleading in 33 per cent and informative in only 40 per cent.

TABLE III. RESULTS IN CASES OF INTRAHEPATIC OBSTRUCTION DUE TO HEPATITIS

CASE	SEX	AGE (YEARS)	DURATION OF JAUNDICE	SERUM BILIRUBIN		B.S.P.		THYMOL TURBIDITY	CEPHALIN CHOLESTEROL (24 HOURS)	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	ACTUAL (%)	CORRECTED (%)			
43	F	57	5 years	1.1	2.1	35	34	10	3+	Operation
44	M	50	4 weeks	1.2	2.4	17	15	15	3+	Clinical data
45	M	21	1 week	1.3	2.1	25	23	7	4+	Clinical data
46	M	51	4 weeks	1.3	2.3	17	14	15	2+	Clinical data
47	M	33	4 weeks	2.1	4.5	14	6	6	1+	Clinical data
48	F	20	1 week	2.1	4.3	41	32	19	3+	Clinical data
49	F	17	1 week	2.3	3.3	60	50	10	3+	Clinical data
50	M	67	2 weeks	3.7	6.5	44	27	6	4+	Clinical data
51	F	25	1 week	3.9	5.5	11	0	23	4+	Clinical data
52	F	62	2 weeks	4.3	8.0	7	0	1	0	Biopsy
53	F	46	2 weeks	4.8	8.6	45	27	22	4+	Clinical data
54	M	54	3 weeks	4.9	8.9	52	33	19	4+	Clinical data
55	F	77	2 weeks	5.6	9.7	23	0	20	4+	Necropsy
56	F	40	6 weeks	6.0	10.0	42	20	29	3+	Clinical data
57	F	45	2 weeks	6.1	9.9	58	23	17	4+	Clinical data
58	M	20	1 week	7.0	11.0	44	21	3	3+	Clinical data
59	F	48	2 weeks	7.0	11.0	46	23	27	4+	Clinical data
60	M	69	2 weeks	7.0	11.0	34	11	11	4+	Clinical data
61	F	50	4 weeks	7.8	13.4	58	33	8	4+	Operation
62	F	66	2 weeks	8.4	14.0	40	14	14	4+	Clinical data
63	F	29	1 week	9.4	12.7	50	23	14	4+	Clinical data
64	M	66	3 weeks	12.4	19.9	52	22	6	2+	Clinical data
65	M	77	2 weeks	13.5	24.0	42	11	21	4+	Clinical data
66	F	42	8 weeks	14.0	25.0	76	44	18	4+	Operation
67	F	66	12 weeks	14.0	24.0	40	8	15	4+	Clinical data
68	F	56	4 weeks	18.0	31.5	38	3	13	4+	Clinical data

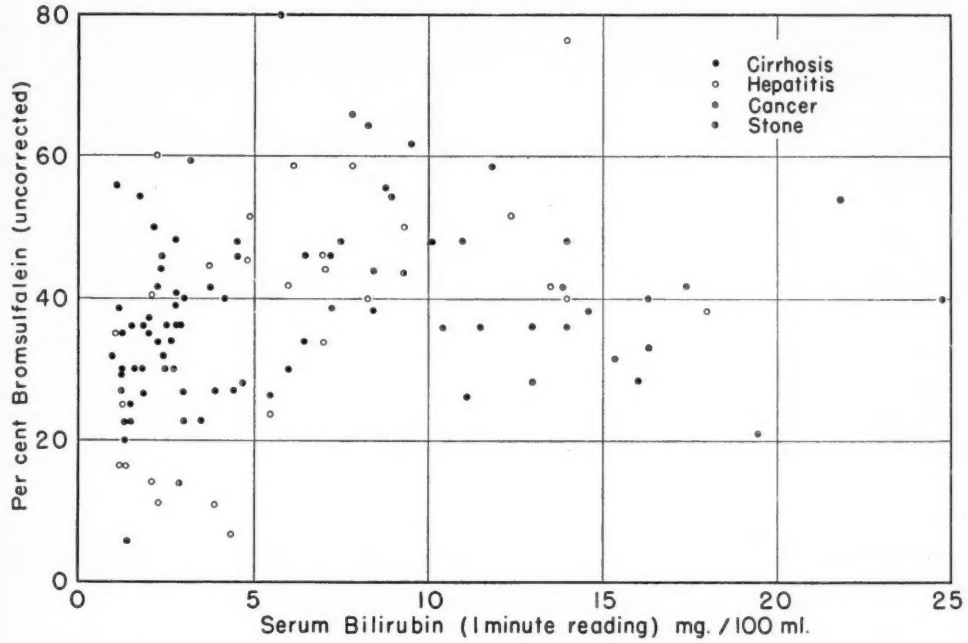

Fig. 1.—Bromsulfalein retention (5 mg. per kilogram, 45-minute reading) in 111 jaundiced patients. The observed (i.e., uncorrected) B.S.P. value has been plotted against the level of the 1-minute serum bilirubin.

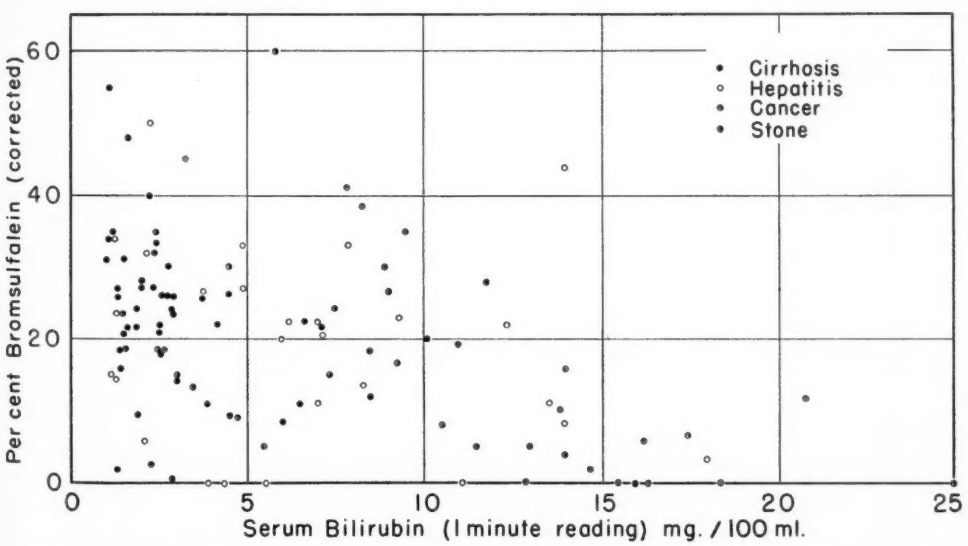

Fig. 2.—Bromsulfalein retention (corrected) plotted against the 1-minute serum bilirubin value in 111 jaundiced patients.

TABLE IV. RESULTS IN CASES OF INTRAHEPATIC OBSTRUCTION DUE TO CIRRHOSIS

CASE	SEX	AGE (YEARS)	DURATION OF JAUNDICE	SERUM BILIRUBIN		B.S.P.		THYMOL TURBIDITY	CEPHALIN CHOLESTEROL (24 HOURS)	DIAGNOSIS ESTABLISHED BY
				1′	TOTAL	ACTUAL (%)	CORRECTED (%)			
69	M	71	Unknown	1.0	2.3	32	31	1	3+	Necropsy
70	F	56	Unknown	1.1	2.1	35	34	10	3+	Biopsy
71	M	58	1 week	1.1	3.5	56	55	12	4+	Necropsy
72	M	49	4 weeks	1.2	2.2	38	35	8	3+	Peri'scopy
73	M	20	3 weeks	1.3	4.0	29	26	28	4+	Biopsy
74	F	38	16 weeks	1.3	3.2	30	27	7	2+	Clinical data
75	F	14	48 weeks	1.4	2.6	6	2	10	4+	Biopsy
76	M	70	6 weeks	1.4	5.3	20	16	12	4+	Necropsy
77	F	56	Unknown	1.5	3.0	25	21	9	4+	Necropsy
78	M	60	6 months	1.5	2.9	23	18	5	2+	Necropsy
79	M	40	1½ years	1.5	4.7	36	31	4	4+	Biopsy
80	F	66	2 years	1.7	3.2	30	22	8	2+	Biopsy
81	M	60	Unknown	1.7	3.7	54	48	7	4+	Necropsy
82	M	59	Unknown	1.9	4.5	36	24	9	4+	Necropsy
83	M	55	Unknown	1.9	3.2	30	22	3	tr.	Necropsy
84	F	41	8 years	1.9	3.7	17	9	5	0	Clinical data
85	F	23	28 weeks	2.0	3.6	37	28	13	4+	Necropsy
86	M	31	Unknown	2.0	3.7	35	27	2	0	Clinical data
87	M	62	Unknown	2.2	4.8	50	40	7	4+	Necropsy
88	F	25	24 weeks	2.3	3.7	34	27	23	4+	Biopsy
89	M	75	24 weeks	2.4	5.2	44	33	13	4+	Necropsy
90	M	55	2 years	2.5	4.5	32	22	6	4+	Necropsy
91	M	61	Unknown	2.5	5.5	36	21	5	4+	Clinical data
92	M	15	1½ years	2.7	4.9	29	17	31	4+	Biopsy
93	M	65	2 weeks	2.7	4.6	48	26	6	4+	Necropsy
94	F	55	8 years	2.8	5.2	42	30	6	1+	Clinical data
95	F	54	8 weeks	2.8	5.1	38	26	8	4+	Necropsy
96	F	55	1 year	2.8	5.9	36	24	15	3+	Clinical data
97	F	18	2 weeks	2.9	4.8	36	23	15	4+	Clinical data
98	M	63	2 weeks	3.0	6.9	40	26	8	3+	Clinical data
99	F	38	3 years	3.0	5.3	27	14	7	0	Biopsy
100	F	61	1 year	3.7	6.4	42	26	14	3+	Biopsy
101	M	63	3 weeks	3.9	7.9	27	11	15	4+	Biopsy
102	F	44	44 weeks	4.2	8.0	40	22	9	0	Clinical data
103	M	65	28 weeks	4.5	7.5	46	26	9	3+	Clinical data
104	F	19	5 years	4.5	8.7	27	9	4	4+	Operation
105	F	47	4 years	6.0	11.1	30	8	14	2+	Necropsy
106	M	27	5 years	6.5	10.6	34	11	11	4+	Biopsy
107	F	9	36 weeks	7.2	13.7	46	22	5	4+	Clinical data
108	M	43	5 weeks	8.5	13.8	38	12	2	4+	Clinical data
109	F	54	1½ years	10.1	17.7	48	20	15	4+	Necropsy
110	F	66	4 years	11.1	18.0	26	0	12	3+	Necropsy
111	M	18	2 years	16.0	23.0	28	0	2	1+	Biopsy

TABLE V. METHOD OF EVALUATION OF TESTS

LAB TEST	SITE OF OBSTRUCTION	RESULTS MAY BE: INFORMATIVE	EQUIVOCAL	MISLEADING
Cephalin Cholesterol Flocculation (24 hours)	Intrahepatic	3+-4+	2+	0 -1+
	Extrahepatic	0 -1+	2+	3+-4+
Thymol Turbidity	Intrahepatic	>7 units	5, 6, 7 units	<5 units
	Extrahepatic	<5 units	5, 6, 7 units	>7 units
Bromsulfalein (corrected)	Intrahepatic	>20%	11-19%	<10%
	Extrahepatic	<10%	11-19%	>20%

TABLE VI. CORRECTED BROMSULFALEIN COMPARED WITH CEPHALIN-CHOLESTEROL AND THYMOL TURBIDITY

	INFORMATIVE			EQUIVOCAL			MISLEADING		
	B.S.P.	C.C.	T.T.	B.S.P.	C.C.	T.T.	B.S.P.	C.C.	T.T.
Hepatitis (26)	15	22	20	5	2	4	6	2	2
Cirrhosis (43)	30	32	25	7	4	11	6	7	7
Total—69	45	54	45	12	6	15	12	9	9
	66%	78%	65%	17%	9%	22%	17%	13%	13%
Cancer (26)	14	16	20	6	7	4	6	3	2
Stone (16)	3	14	11	5	1	3	8	1	2
Total—42	17	30	31	11	8	7	14	4	4
	40%	71%	74%	26%	19%	16%	33%	10%	10%

DISCUSSION

For clinical purposes, the bromsulfalein test is unquestionably a useful means of assessing liver function. In the absence of jaundice, the procedure is reliable and the results are reproducible. The availability, simplicity, and relative freedom from untoward sensitivity reactions assure this procedure a place as a dependable laboratory test. The actual mechanisms involved in B.S.P. extraction and excretion are complex and poorly understood. The blood supply to the liver and particularly the rate of blood flow are recognized as important factors in governing extraction rates. Andrews,[8] in his recent paper, has stressed these aspects of B.S.P. removal. If, in addition, one adds partial or complete extrahepatic biliary duct system obstruction, the mechanisms involved in B.S.P. extraction and excretion become even more complex. It is very difficult to test the validity of the correction formula proposed by Zieve and associates. Since it was derived from clinical data, one means of testing its clinical *usefulness* is to apply it to the problem of diagnosis in other cases of liver disease. This has been done in the current study.

The limitations of the so-called hepatic tests employed in clinical practice are well known. Despite the handicaps that their lack of precision entails, the cephalin-cholesterol flocculation test and the thymol turbidity reaction are useful in the problem of the differential diagnosis of jaundice. The results, as indicated in Table VI, were informative in 65 to 78 per cent of instances. While such performance is far from ideal, this appears to be the best that can be obtained at present. The B.S.P. test in this series of 111 jaundiced patients was less informative than that of the two more simple tests in the problem of differential diagnosis.

In discussing the limitations of the corrected B.S.P. test, Zieve and associates[6] indicate that the procedure may be unreliable in patients with severe jaundice of short duration. The duration of jaundice in our patients is indicated in Tables I to IV. It is apparent that in many instances jaundice had been present for an appreciable period of time. Thus the B.S.P. test was not performed at a time when the level of bilirubin in the blood was rapidly changing.

The present study has not actually tested the validity of the proposed correction factor for the performance of the bromsulfalein test in the jaundiced patient. We have merely sought to answer the question: Does the information gained by performing the test in a jaundiced patient serve a useful clinical purpose? It may be argued that many of the patients with stone or cancer obstructing the bile ducts did have functional (and structural) impairment and that the abnormal retention of bromsulfalein was a true reflection of this derangement. It may be contended that the thesis: "jaundice with abnormal hepatic tests suggests hepatocellular disorder and jaundice with normal hepatic tests suggests extrahepatic obstruction" is an oversimplification of a complex problem. Nevertheless this does represent a philosophy that has been utilized in clinical practice for some time and doubtless will continue to be used because it helps solve problems in differential diagnosis. The results of the corrected bromsulfalein test have been evaluated by this simple criterion. It has been found to be less helpful than the cephalin-cholesterol flocculation test or the thymol turbidity test.

CONCLUSIONS

1. The bromsulfalein test was performed in 111 jaundiced patients. The "correction factor" proposed by Zieve, Hanson, and Hill was applied. The results of the test in patients with intrahepatic disease (cirrhosis or viral hepatitis) were compared with those in patients with extrahepatic obstruction (cancer or common duct stone).

2. Poor correlation between the level of the serum bilirubin, the degree of dye retention and the type of disorder present was observed. The "corrected" B.S.P. test offered less assistance in the differential diagnosis of jaundice than did the cephalin-cholesterol flocculation test or the thymol turbidity test.

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OBSERVATIONS ON THE CYTOLOGY OF GASTRIC EPITHELIUM IN TROPICAL SPRUE

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INTRODUCTION

RECENT reports[1, 2] have indicated abnormal cellular changes in the squamous epithelial and gastric columnar cells of patients with pernicious anemia. Such cytological changes appear to be most marked during relapses of the disease and may persist for many years after the patient has received adequate antianemic therapy and no longer has macrocytic anemia. In view of the similar hematological changes that exist in tropical sprue, the present study was initiated to determine if squamous epithelial changes could be observed in tropical sprue patients with glossitis. The criteria for the diagnosis of tropical sprue outlined by Rodriguez-Molina[3] were followed in this study. All untreated patients had a macrocytic anemia with a megaloblastic arrest of the bone marrow associated with diarrhea, glossitis, weight loss, and weakness. No consistent abnormalities could be detected, however, by simple scrapings of the tip and border of the tongue. The lack of squamous cellular changes may be associated with the paucity of severe glossitis found in tropical sprue observed in Puerto Rico at the present time.

A more complete study of the stomach epithelium was begun, therefore, with utilization of the lavage and the hairnet balloon technique.[4] Both of these procedures were time consuming[5] and debilitating to the patient with severe anemia. Since the studies were done in hospitals at a distance from the laboratory, immediate centrifugation and preparation of the collected specimens were not possible. Efforts to preserve stomach lavage material by chilling for several hours were not satisfactory. Such procedures yielded large quantities of squamous epithelial cells and only rare columnar cells. Therefore, the Ayre gastric brush was procured for further study.[6] This technique has been used over a hundred times without complication. The brush can be passed and a sample obtained in less than ten minutes. It was evident early in the study that the brush sampling produced adequate cellular material.

MATERIALS AND METHODS

Patients with a clinical and laboratory diagnosis of tropical sprue were studied during relapse and remission. All such patients had a megaloblastic bone marrow at the time of relapse. In a few instances some patients were studied during the initial treatment period with folic acid or vitamin B_{12}. When possible free gastric acidity was determined

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only as to presence or absence after histamine stimulation. If patients did not have free acid after a food or alcohol test meal, the results were listed as not done (N.D.), inasmuch as maximum stimulus with histamine was not used. To compare with the tropical sprue patients, a series of examinations was performed on twenty-two volunteer patients who had no gastrointestinal symptoms.

The patients' pharynxes were anesthetized with 0.25 per cent tetracaine hydrochloride solution. When the posterior pharynx could be touched with a tongue blade, adequate anesthesia existed to pass the gastric brush. Holding the tongue forward with one hand, the metal tip of the gastric brush was inserted into the hypopharynx and gently pushed into the esophagus. It was found that the tip was passed more easily if the distal lumen opening was covered with half of an empty No. 000 gelatin capsule. The plastic tubing was pushed gently to a 27 cm. mark on the plastic tubing at a level with the lips. The brush was extended from the lumen and turned vigorously as the plastic tubing was slowly pulled out of the mouth to a 17 cm. mark on the tubing. At this level the brush was pulled inside the metal tip and the plastic tube removed from the stomach. The entire procedure usually requires less than five minutes from the time that one begins to insert the tubing. The brushings were done between a measured distance on the tubing in an effort to collect material from as complete an area of the stomach mucosa as possible. One must recognize that there is no method with this technique to evaluate what area of the stomach is represented by the cellular material collected. In patients of small stature, the plastic tubing could not be inserted to the 27 cm. mark. No fluoroscopic studies were done to locate the brushed area of mucosa.

Cellular material was scraped from the brush fibers with the end of a clean glass slide. Portions of the mucoid cellular material were then transferred to slides coated with Mayers' albumin. A portion of the cellular material was carefully spread between two coated slides in a fashion similar to the preparation of blood films and fixed in alcohol-ether solution for at least one hour. Twelve slides were made, if possible, from each specimen. They were stained by the method of Papanicolaou.[7]

In some instances the size of nuclei in columnar cells was measured with a calibrated eyepiece under oil immersion magnification. Columnar cells were measured in which no distortion of the outline existed from preparation. The widest diameter of the nucleus was measured and charted in the fashion of a Price-Jones Curve as described by Graham and Rheault.[1]

RESULTS

Cellular material derived from the bristles of the brush for the most part consist of sheets or clumps of cells. The abrasive action of the bristles apparently peels away small groups of columnar cells. Usually the mucoid material on the brush has been small fragments which could be spread by gentle pressure between glass slides. In such manipulations some cells will be distorted and areas of the slide must be examined in which the cells have not been torn or elongated. Numerous squamous epithelial cells are present from normal pharyngoesophageal desquamation. The passage of the metal tip usually will produce sheets of squamous cells to allow adequate material for study.

Some difficulty has been encountered in variability of tinctorial intensity of the Papanicolaou stain. These variations appear to be associated with the high humidity of Puerto Rico, where the study was done. Therefore, interpretations have been made on the morphologic changes of the epithelial cells rather than on variation in staining qualities of the cytoplasm.

Twenty-two patients without gastrointestinal disease were studied for control observations. No abnormalities were noted in the squamous epithelial cells. These cells presented the classical appearances ascribed to squamous epithelium; large, flat cells with clear cytoplasm and a small pyknotic central nucleus (Fig. 1). There was marked variation in the size of such cells in these preparations. The majority of cells ranged from 15 to 25 μ in diameter. If large numbers of cells were studied, a rare binucleated cell could be found. About a third of the squamous cells have some reticular network to the dark nucleus if examined under oil immersion magnification.

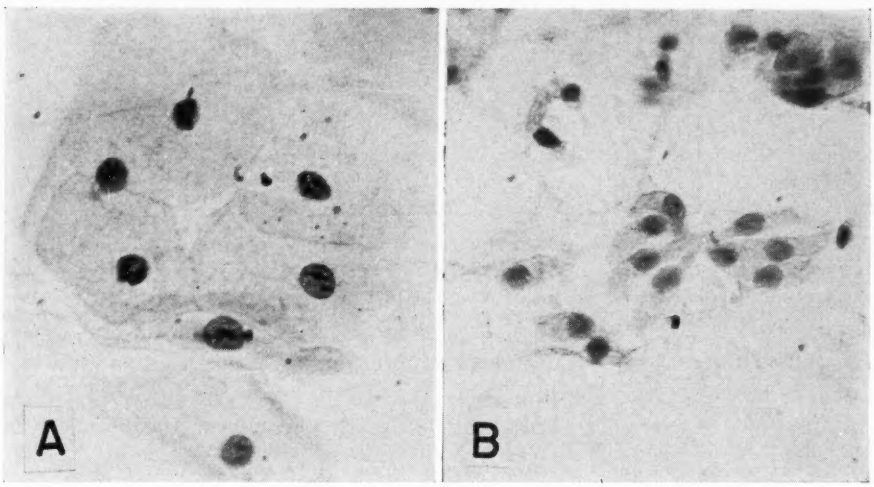

Fig. 1.—Gastric epithelial cells. (×750; reduced ¼.) *A*, Normal squamous cells from nasopharyngeal desquamation. The pale cytoplasm has a central pyknotic nucleus. A faint outline of nuclear chromatin can be seen in one cell. *B*, Normal gastric columnar cells. The darkly stained nucleus is eccentric in location. The pale cytoplasm has a granular appearance and an indistinct border.

The normal columnar cell has a much more descriptive cytology. The cytoplasm is pale and many times only a faint irregular outline of the border can be detected. There are subtle variations in the appearance of the cytoplasm to suggest small vacuoles or irregularity of staining. Marked variations exist on the shape and contour of columnar cells. The ovoid nucleus is eccentric in location and the chromatin material stains darkly and is evenly distributed (Fig. 1). In view of the difficulty in estimating the total length of columnar cells, nuclear size was measured as the widest diameters of one hundred consecutive cells. Only cells that were well defined and in small clumps were measured. The mean curves from 7 control patients are plotted in Fig. 2. Several preparations from the same aspiration were measured at different times to evaluate the reproducibility of such measurements, and the results did not perceptibly alter the initial values.

Among the volunteer patients without gastrointestinal disease three were observed in whom the columnar cells were slightly larger than had been expected. There were no marked changes in the nuclear pattern. Two of these

patients had evidence of malnutrition and moderate weight loss. Whether these changes reflect early columnar cell changes of nutritional deficiency cannot be evaluated with certainty at this time.

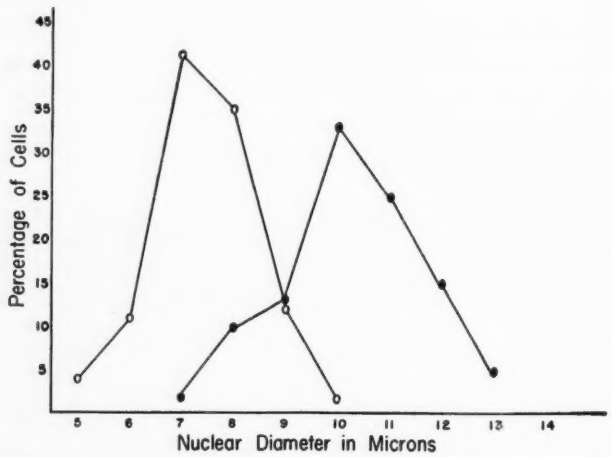

Fig. 2.—Nuclear diameters of gastric columnar cells. The open circles represent the mean curve of seven control patients. The closed circles represent the mean curve of 4 patients with tropical sprue. In each preparation a hundred columnar cells were counted.

Nineteen untreated patients with tropical sprue were studied before any antianemic therapy was begun (Table I). However, some patients had been in the hospital for seven to ten days before the cytology studies were done.

TABLE I. UNTREATED SPRUE PATIENTS

CASE	AGE	GASTRIC ACIDITY	EPITHELIAL CELLS*					
			SQUAMOUS		COLUMNAR			
			DN	AN	PC	DN	SC	P
1	59	0	+	+	+			
2	32	+	+	+	+		+	
3	77	+	+	+	+	+	+	
4	64	N.D.	+		+	+	+	+
5	19	+	+	+	+		+	
6	37	+	+					
7	46	+	+	+	+	+	+	
8	58	N.D.	+	+	+	+	+	+
9	68	N.D.	+	+			+	+
10	38	+	+		+			
11	67	N.D.	+	+	+		+	
12	65	+	+	+	+	+	+	+
13	33	+	+		+	+		
14	72	+	+	+	+	+	+	+
15	51	+	+	+	+	+	+	+
16	60	+	+	+	+	+	+	+
17	57	+	+	+	+		+	
18	26	+	+	+	+	+	+	+
19	72	N.D.	+		+	+		

Cytological observations on the nuclear changes of squamous and columnar cells. Only positive observations are tabulated. Gastric acidity is listed as not done (N.D.) if histamine stimulation was not used.

*Abbreviations as noted in text:

DN, Double nuclei.
AN, Abnormal nuclei (bizarre pattern, karyorrhexis).
PC, Pale cell (large open nucleus with irregular clumps of chromatin).
SC, Serrated cell (irregular nuclear border with foldings of the chromatin pattern).
P, Phagocytosis (inclusion of polymorphonuclear cells in the cytoplasm of columnar cells).

In some instances the improved nutrition of a hospital diet initiated a clinical response that may have been associated with a hematological (reticulocyte) response without specific therapy. In this present study one sampling of gastric material was collected before therapy. Interpretations therefore reflect a single sampling. Possibly additional data could be obtained if the procedure had been repeated several times.

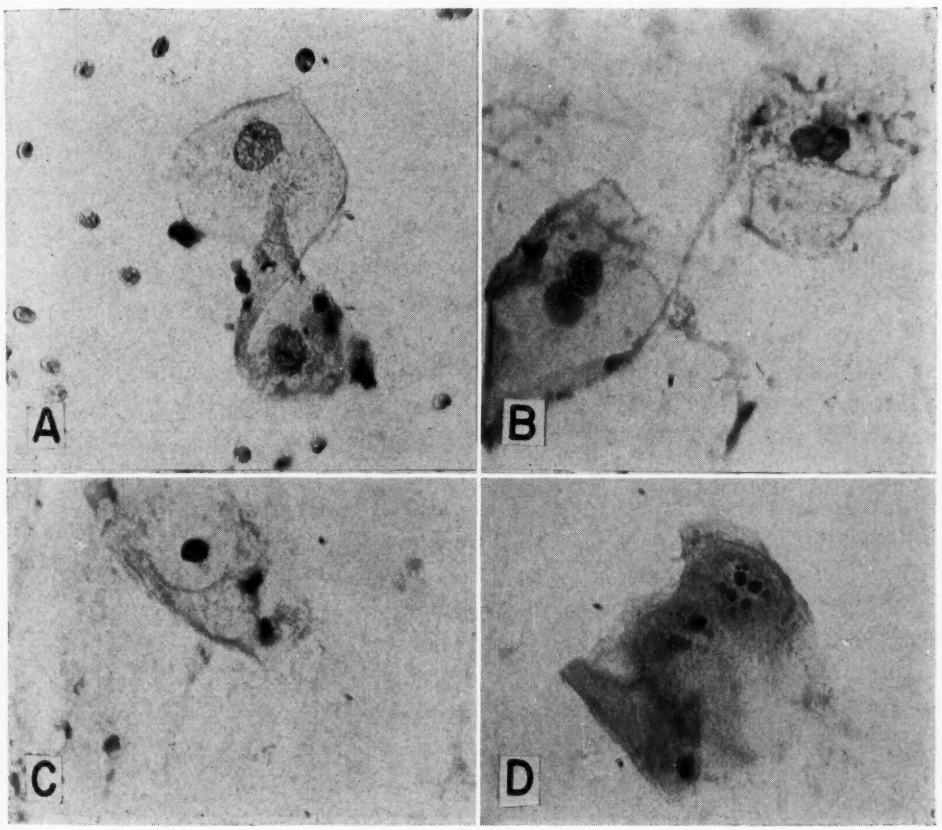

Fig. 3.—Abnormal squamous cells observed in untreated tropical sprue. (×750; reduced ¼.) *A*, Large, pale nucleus with dissolution of compact chromatin pattern. *B*, Abnormal nuclear division. The lower cell has an incomplete division of the nucleus. In some instances the irreguiar nucleus may show four or five "buddings." The upper cell has two nuclei. These nuclear changes are observed most frequently. *C*, A circular halo around a pyknotic nucleus. This variation is seen frequently and may represent disintegration of the nucleus. *D*, Karyorrhexis.

All such patients had abnormal squamous epithelial cells present. The nucleus had an open network to the chromatin pattern that is not observed in the normal cell with a pyknotic nucleus (Fig. 3, *A*). There was marked increase in the multinucleated cells. The majority of such cells were binucleated (DN) and had the appearance of two kidney beans partially overlying each other (Fig. 3, *B*). Other cells showed a nuclear fragment at some distance from the center. Associated with the multinucleated cells, other variations of the nuclear pattern existed. Nuclear fragments were found at a distance from the nucleus. Some cells had an irregular serrated outline of the nucleus that

indicated partial or abnormal division (AN). Frequently a pale area around the nucleus suggested a punched-out area of the cells under low magnification (Fig. 3, *C*). Karyorrhexis was present (Fig. 3, *D*). There was wide variation in the size of squamous cells obtained with the gastric brush. No correlation existed between the total size of cell and nuclear abnormality. More abnormal nuclear patterns were observed in large squamous cells in untreated patients. Such patients also showed a larger variation in the size of the squamous cells than was noted in control patients. These cytological changes appear to be similar to the abnormal squamous cells in pernicious anemia described by Graham and Rheault.[1]

The other distinctive and diagnostic cytological variation is associated with the columnar cells. Excellent preparations are obtained with this procedure, for the abnormal columnar cells in the untreated sprue patient tend to separate more easily than normal cells. The cytology of the columnar cells appears to be similar to those changes described previously in pernicious anemia by Massey and Rubin.[2] The columnar cell on inspection was larger in width and length. However, the indistinct irregular border of the cytoplasm made it difficult to compare the size of the cell accurately with the normal. When the cytoplasm was stained deeply it appeared to be more granular. Some cells had inclusions which may represent abnormalities of nuclear material. In some instances the nuclear outline of polymorphonuclear granulocyte inclusions could be observed (P) (Fig. 4, *C*). It was concluded that some of the inclusion fragments in the cytoplasm represented dissolution of these white cells. Such changes have not been described in the gastric cytology of pernicious anemia. No phagocytosis of white cells was observed in the columnar cells in the normal volunteer patient.

The size and changes in the nucleus of the columnar cells were the most outstanding variation in the sprue patients. In contrast to the darkly stained nucleus of the normal columnar cells, these cells were larger and had a pale background with irregular clumping of the chromatin material (PC) (Fig. 4, *A*). The chromatin tended to form several aggregates with a fine, irregular network interlacing through the nucleus. The nuclear borders of some columnar cells were irregular and serrated (SC). Some cells had a greater tendency for folding and interweaving the nuclear membrane. The large, pale nucleus described above could exist without these border changes (Fig. 4, *B*). However, the serrated border always was present with the abnormal pale nucleus. A Price-Jones type of curve for comparison with the normal columnar cell was determined by counting a hundred nuclei of these large cells in preparations of four patients. A mean curve is presented in Fig. 2. There was marked increase in the nuclear size in the tropical sprue patient.

The abnormal nuclear development was also associated with bizarre cell division in the columnar cells. As many as four distinct nuclei were observed in one cell. However, it was more common to see two nuclei, often incompletely divided (DN). Such abnormal division likewise may be frequent in

one area of a smear and absent on several other smears. These limited areas suggest that some areas of gastric mucosa may have more abnormal cells than others. There was marked variation in the number of the abnormal cells observed as the slides were studied. Some smears showed almost a total loss of normal cellular cytology. Another smear may show a predominance of normal

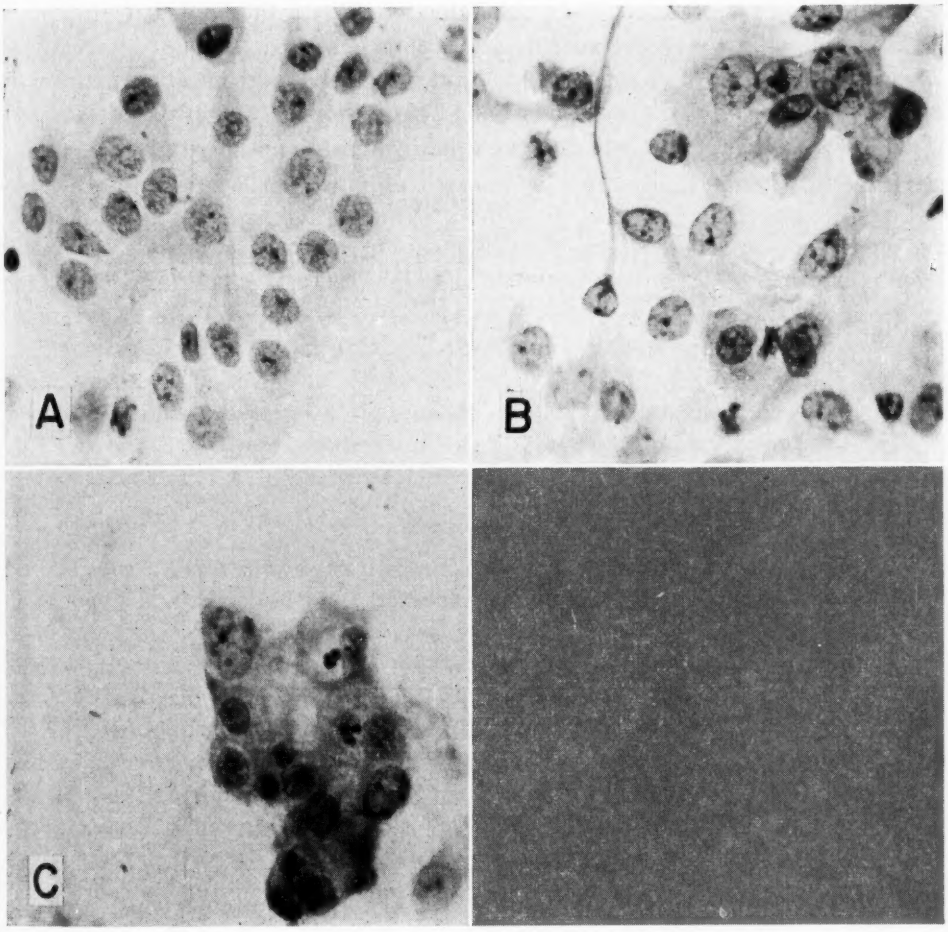

Fig. 4.—Abnormal gastric columnar cells observed in tropical sprue. (×750; reduced ¼.) *A*, Usual preparation obtained with gastric brush procedure. The cytoplasm is pale and has an indistinct outline. The nuclear changes among this group of cells represent early changes. The pattern is lacy and a few small clumps of chromatin are present. *B*, More advanced pale cell type with irregular chromatin clumping. One may note the marked increase and variation of size of nucleus when compared with Fig. 1. The irregular border with serrated cell type is not well shown, although one cell in lower middle portion has a folding of the nucleus to suggest abnormal division. *C*, Leukocyte inclusions. The halo around neutrophil at upper border of cell clump suggests chromatolysis. Several other leukocytes can be observed in this group of columnar cells.

columnar epithelium, with only a few abnormal cells interspersed among normal elements. Hence it is of value to study numerous slides to obtain full evaluation of the gastric epithelium. A review of the columnar cell alterations would indicate that the untreated sprue patient has cytological changes as seen in pernicious anemia. Multinucleated columnar cells as noted here

have been described.[1] The "bland cell type" with a serrated nuclear border and the "P.A. cell type," as described by Massey and Rubin,[2] are the predominate columnar cell abnormalities observed in these patients.

In Table I a listing of the abnormal findings is tabulated. In view of the variations noted as numerous slides of one sampling were studied, the frequency of cell types described is not indicated. With the present technique it is advisable only to list the specific alterations observed. It is of interest that one patient had only changes in the squamous cells, and no abnormalities in the columnar cells in the one study done before therapy was initiated (Case 6). The absence of columnar cell changes would warrant another study.

TABLE II. TREATED SPRUE PATIENTS

CASE	AGE	THERAPY†	DURATION OF THERAPY	GASTRIC ACIDITY	EPITHELIAL CELLS*					
					SQUAMOUS		COLUMNAR			
					DN	AN	PC	DN	SC	P
1	59	B_{12}	5 mo.	0			+			
2	32	F.A.	2 mo.	+			+	+		
3	77	B_{12}	5 mo.	+	+	+	+			
4	64	B_{12}	4 mo.	N.D.	+	+				
5	19	F.A.	2 mo.	+	+	+	+		+	+
6	37	F.A.	5 mo.	+						
7	46	F.A.	12 days	+	+		+		+	
7A	46	F.A.	3 mo.	+						
8	58	B_{12}	2 wk.	N.D.	+	+	+	+	+	+
9	58	F.A.	2 yr.	+	+	+	+		+	
10	50	F.A.	2 yr.	+			+		+	+
11	84	F.A.	2 yr.	+			+	+		+
12	78	F.A.	2 yr.	+						
13	72	B_{12}	1½ yr.	0			+	+	+	+
14	39	F.A.	1½ yr.	N.D.						
15	39	F.A.	5 yr.	+			+			
16	60	B_{12}	3 yr.	+			+		+	+

Cytological observations during specific therapy. The first eight cases listed represent follow-up studies on patients who were examined before treatment initiated. Compare with Cases 1 to 8 in Table I.

*Abbreviations are similar to Table I.

†Therapy:

F.A. = Folic acid — 5 mg. daily, orally.
B_{12} = Vitamin B_{12} — 30 mcg. weekly, parenterally.

Sixteen patients were studied after they had received orally 5 mg. of folic acid daily or 30 mcg. of vitamin B_{12} weekly (intramuscularly) for the intervals of time listed in Table II before cytological studies were done. With such a treatment program the macrocytic anemia disappeared in all patients receiving therapy for one month or longer. The first eight patients listed in Tables I and II represent gastric cytological studies done before and during therapy. The cytological changes did not disappear rapidly with specific antianemic therapy as did the megaloblastic changes of the bone marrow. Squamous cell changes during therapy primarily were associated with the continued presence of double nuclei. The vast majority of the cells had normal pyknotic nuclei and the nuclei were not large even in the presence of abnormal columnar cells. In patients who were studied after short periods of treatment (Cases 3, 4, 5, 7, 8), more abnormal squamous cell abnormalities persisted.

Squamous cell changes were not observed in patients who had prolonged therapy except in one patient (Case 9), who had a good hematological remission.

The abnormal gastric cell pattern observed in the untreated patients persisted. Some columnar cells had inclusions which were fragments of granulocytes; some cells showed clearly mature polymorphonuclear white cells phagocytized in the cytoplasm. Associated with these inclusions, numerous white cells were present on the smears to suggest infiltration of the mucosa. Gastric biopsies would be an aid to determining if a leukocytic infiltration of the mucosa does exist. The nuclear patterns described above in untreated patients also were present in this group. In Table II the presence or absence of such cell changes is tabulated. However, in the examination of numerous smears in each study, it was apparent that columnar cells with such nuclear changes were less frequent than in the untreated patients.

While abnormal columnar cells persisted, numerous areas containing normal columnar cells were observed. There was, therefore, a mixed population noted after treatment. Efforts were made to grade the cellular variations with duration and type of therapy. However, the variation among columnar cells in the study of numerous slides from individual patients emphasized that the collection technique or cytological variation in stomach areas prevented an adequate estimation of the degree of mixed columnar cell alteration in the treated sprue patients.

DISCUSSION

These gastric cytological observations were made on patients who had macrocytic anemia and megaloblastic bone marrow alterations before specific therapy was given. In contrast to pernicious anemia in which similar cytological changes have been described, in most instances these patients had free gastric acidity. Likewise, there is no evidence to demonstrate a defect of intrinsic factor function. Hematological abnormalities have indicated an inadequate erythropoietic nutritional source of folic acid or vitamin B_{12}. The squamous cells in the majority of treated sprue patients reverted to a normal appearance. Therefore the cytological abnormality of this cell is more closely correlated with the nutritional defect of the bone marrow. This observation has also been noted in pernicious anemia.[1] Minimal squamous cell alterations persisted in only three patients who had received treatment for at least three months. At the present time there is no clinical evidence to suggest that these patients had a poorer response to therapy or an incomplete hematological remission to explain these persistent changes.

The reason for the continued cytological alterations in the columnar cells of treated sprue patients is obscure, for all patients had a good hematological remission. Oral absorption tests and fat balance studies have demonstrated a marked impairment in the assimilation of sugars, fats, and vitamins in sprue patients.[8] Many of these absorption defects persist during prolonged treatment with folic acid or vitamin B_{12}. Although adequate folic acid is absorbed, other nutritional factors may be causative agents for the abnormal cytology. In the present study adequate therapy for control of anemia and glossitis was

utilized. Since the columnar cell abnormality persisted after adequate vitamin therapy for a hematological response, one may suspect that other nutritional factors are necessary.

An alternate explanation may be related to the duration of tropical sprue. The disease is associated with many episodes of intermittent diarrhea before therapy is initiated. One patient (Case 7) had progressive improvement in the cytological changes during a four-month period of folic acid therapy. Careful questioning revealed no previous history of diarrhea or lassitude to suggest that the patient had incipient tropical sprue for a long period. As noted in Table II no abnormal cells were noted after three months of folic acid therapy. Further investigation will be needed to determine if irreversible changes in the gastric cytology are associated with duration of tropical sprue. The continued absorption defects of the small bowel after therapy does indicate that some small bowel functions are abnormal despite prolonged folic acid and vitamin B_{12} administration.

Klayman and Massey[9] have demonstrated recently similar columnar cell changes in patients with achlorhydria in the absence of pernicious anemia. Such studies have emphasized that the abnormalities were quite comparable to the "bland cell type" of columnar cell described by Massey and Rubin. Probably this type of cytological alteration can be considered to be associated with generalized mucosal atrophy. In contrast to such observations, the abnormal columnar cells in tropical sprue patients were seen in the presence of free gastric acid and in all age groups (Case 5 was 19 years old). Rodriguez Olleros,[10] in 1938, described the gastric mucosa of 28 tropical sprue patients by gastroscopy. Ten untreated sprue patients had atrophy of the gastric mucosa. However, the author indicated that the atrophy changes were much less severe than the changes noted in pernicious anemia. Eight of these patients had adequate free acid by histamine or caffein stimulation. Patients treated with liver extract and free of gastrointestinal symptoms had minimal or no mucosal atrophy. The author concluded that some relationship existed between the anemia and intensity of symptoms and the degree of gastric atrophy. Gastroscopic observations were not made in this present study. However, the presence of free acid and the previous observations of Rodriguez Olleros[10] suggest that the columnar cell changes observed in treated sprue patients may not be associated with atrophy of the gastric mucosa.

The persistence of abnormal columnar cells in the stomach of the treated sprue patient may reflect changes in the epithelium of the small bowel. Autopsy and biopsy material from nontropical sprue patients have demonstrated alterations in the intestinal villi.[11, 12] Certainly, similar studies of the small bowel with procedures as applied in this technique to the gastric mucosa would be helpful in efforts to correlate cellular function with impaired absorption of foodstuffs.

SUMMARY

A cytological study of gastric cells has been conducted in tropical sprue utilizing a simple procedure of collecting mucosal cells with a rotating brush.

Abnormalities of squamous and columnar cells have been observed which appear to be similar to changes associated with pernicious anemia. In contrast to the associated absence of gastric acidity in pernicious anemia, such changes may be found in tropical sprue without achlorhydria. These cellular changes may continue after adequate antianemic therapy with folic acid or vitamin B_{12}.

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MACROGLOBULINEMIA AND ITS RELATIONSHIP TO OTHER PARAPROTEINS

With a Case Report

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PRIMARY macroglobulinemia is a rare chronic disease characterized by the formation of an abnormal serum protein of large molecular weight and associated clinically with ill-defined symptomatology and various hematologic and hemorrhagic manifestations. The disease was first described by Waldenström and most of the cases have originated in the Scandinavian and European countries[1-18]; only recently have two reports been recorded by workers in the United States.[19-20] Secondary macroglobulinemia associated with other disease processes has also been reported. Observations made on a patient with macroglobulinemia, anemia, and thrombocytopenia are herein presented with studies on the associated coagulation defects in which deficiencies in AC globulin and prothrombin and a qualitative abnormality of the fibrinogen were found. The relationship of macroglobulinemia to other paraproteinemias is also reviewed.

CASE REPORT

A. K.,* 79-year-old white woman, was admitted to Barnes Hospital for the first time in April, 1951, for surgical treatment of a left retinal detachment. Her past history included a mastoidectomy 40 years previously, gall bladder symptoms for 30 years, and an acute phlebitis of the right leg in 1948. A normocytic normochromic anemia and leukopenia with relative lymphocytosis were noted in 1951 (see Fig. 1). Occult blood was detected in the stool at that time.

The second admission was in July, 1951, for weakness, decreased appetite and strength, and a 13 pound weight loss of two months' duration. The physical examination was normal except for pallor of the skin and mucous membranes, a soft aortic systolic, and a Grade II apical systolic murmur. The liver, spleen, and lymph nodes were not enlarged. Hematologic studies revealed a normocytic normochromic anemia (see Fig. 1), normal white cell and platelet counts, normal white cell differential, and marked rouleau formation of the erythrocytes. Sternal marrow aspiration yielded relatively hypocellular tissue with scattered foci of lymphocytes, plasma cells, and tissue basophils (see Fig. 2, *A*). Bence-Jones proteinuria and cryoglobulins were absent. The nonprotein nitrogen, fasting blood sugar, and alkaline phosphatase were normal, and the total proteins were 11.6 Gm. per cent, albumin 3.4 Gm. per cent, and globulins 8.2 Gm. per cent. Free acid was present in the gastric contents after histamine injection. Roentgenograms showed minimal cardiac enlargement, calcification in the aortic arch and in the region of the gall bladder, and hypertrophic

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osteoarthritis limited to the lower dorsal and lumbar spines; no bony defects were noted in the ribs, vertebrae, long bones, skull, or pelvis. Cystoscopic examination showed chronic granular urethritis. Chloramphenicol therapy was given for the latter and transfusions of 2,500 ml. whole blood for the anemia.

In December, 1951, and in April, 1952, she again received transfusions. During the latter period, a severe transfusion reaction occurred with development of anuria, uremia, generalized edema, and convulsions; she recovered in three days. Between May, 1952, and October, 1954, the patient noted only weakness and easy fatigability. In November, 1954, bloody vaginal discharge, petechiae, and ecchymoses developed. A marked anemia, thrombocytopenia (see Fig. 1), prolonged bleeding time, and a Lee-White coagulation time of 14 minutes were found. Vitamin K given orally and vitamin B_{12} intramuscularly were ineffective, and the patient was admitted to Barnes Hospital for the third time.

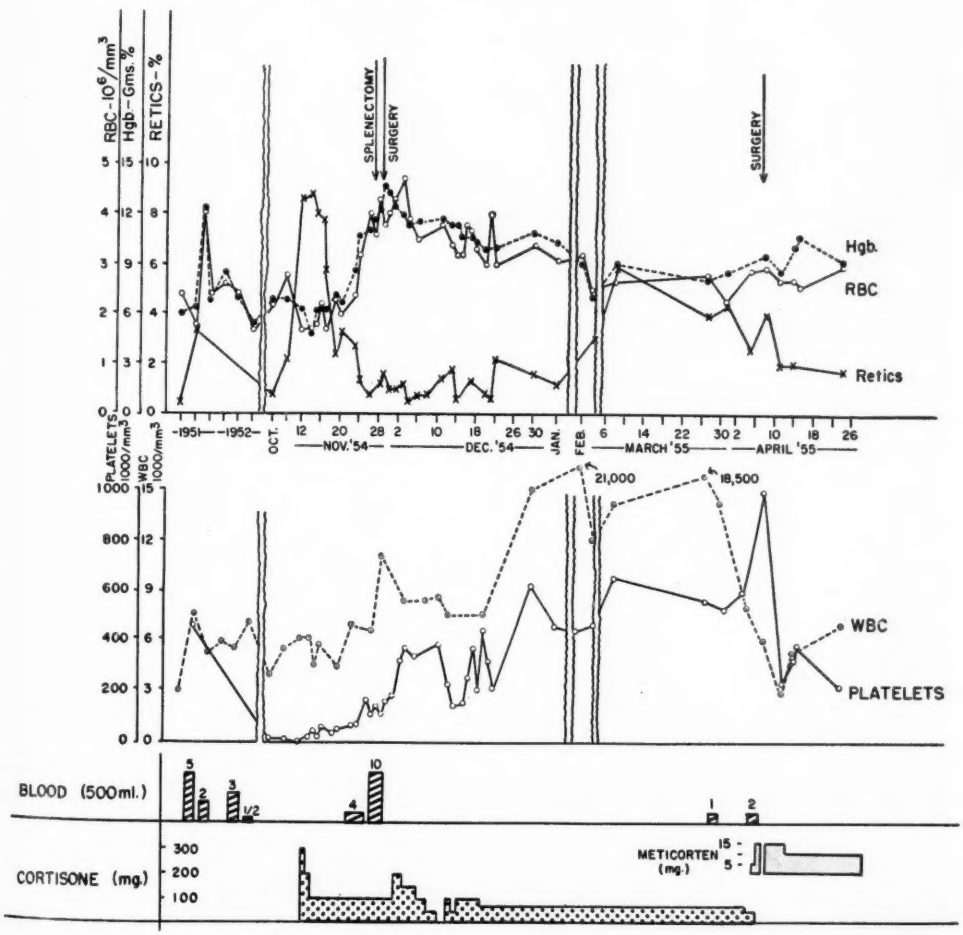

Fig. 1.—Peripheral blood counts and therapy in a patient with macroglobulinemia and thrombocytopenia.

Physical examination was remarkable for the presence of generalized petechiae, ecchymoses, hepatomegaly of 8 cm. below the right costal margin, and splenomegaly of 6 cm. below the left costal margin. Marked rouleau formation of the red cells was again noted, and although the total white blood cell count was within the normal range, differential count showed 18 to 72 per cent lymphocytes and an occasional normoblast in the peripheral blood. Sternal marrow aspiration revealed hypercellularity of all elements, normal myeloid

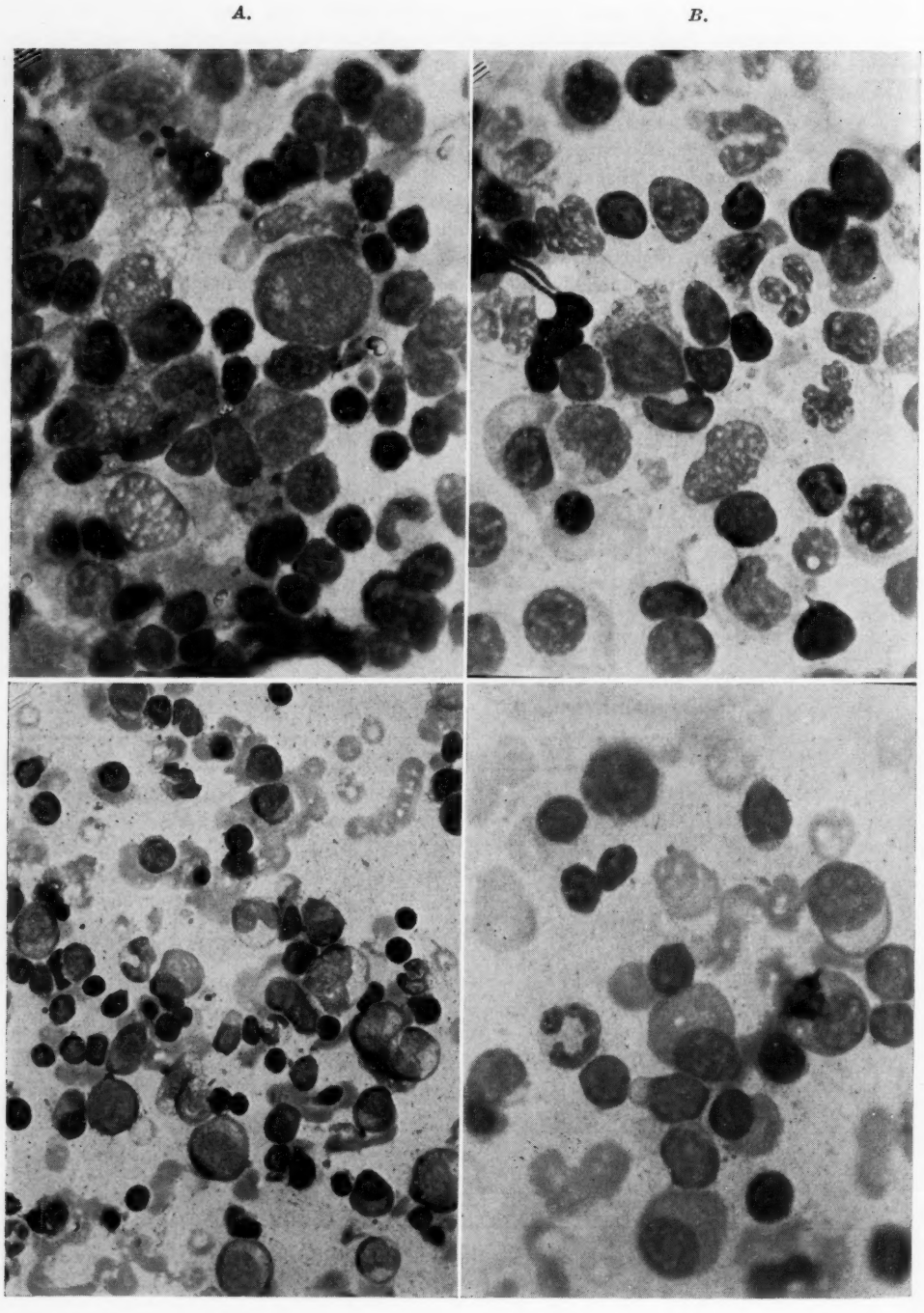

Fig. 2.—Histologic appearance of the sternal bone marrow and spleen. Sternal bone marrow aspirations (Wright's stain): *A*, July, 1951 (×1,170). *B*, November, 1954 (×1,170). *C*, March, 1955 (×600). Spleen: *D*, Touch preparation from the cut surface of the surgical specimen (Wright's stain, ×1,000). *E*, Section of the surgical specimen (H & E stain, ×275). (All reduced ⅛ off.)

and erythroid series, and numerous megakaryocytes in all stages of development but with agranular cytoplasm and absent platelet formation (see Fig. 2, *B*). Urinalysis showed a specific gravity of 1.018, 1+ protein, and 2 to 3 white blood cells per high power field. Other laboratory data included: cephalin cholesterol flocculation 3 plus, thymol turbidity 9.8 units, van den Bergh less than 0.8 mg. per cent, total protein 9.9 Gm. per cent, albumin 2.3 Gm. per cent, globulin 7.6 Gm. per cent, fasting blood sugar 87 mg. per cent, cholesterol 84 mg. per cent, sedimentation rate (Wintrobe method) 68 mm. per hour, indirect Coombs test negative, Rh antibody positive 1:1 titer in saline and 1:32 titer in albumin, and one negative L.E. preparation. A platelet agglutinin determination was unsatisfactory because of lysis of the normal platelet suspension reagent, presumably due to the marked hyperproteinemia. Roentgenograms of the chest, abdomen, and bones were essentially as noted in 1951.

Cortisone administration was followed by disappearance of the petechiae, fading of the ecchymoses, and gradual rise in platelet count (see Fig. 1). On Nov. 27, 1954, the spleen and a small accessory spleen were removed.* Examination of the abdomen also revealed many 1 cm. stones in the gall bladder and edema of the pancreas. Dr. L. V. Ackerman reported the following findings:

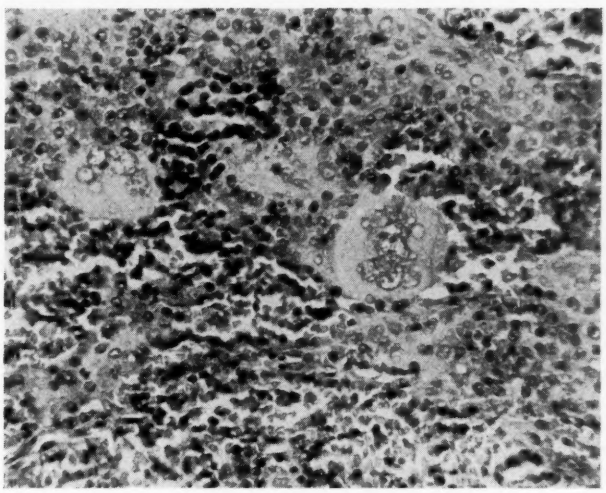

Fig. 2*E*. (*For legend see opposite page.*)

Gross Pathology.—The spleen weighed 490 grams and measured 16 by 10 by 5 cm. The capsule was smooth, of normal thickness, and had a mottled, dark, slate-blue color. Multiple parallel cuts disclosed uniform dark purplish-red surfaces in which trabeculae and Malpighian corpuscles are widely separated, have indistinct margins, do not have active germinal centers, and vary in size, but are generally smaller than normal. There is generalized congestion with many sinusoids widely dilated and partially filled with blood. A diffuse reticulum hyperplasia is seen in all sections. Fibrous trabeculae are indistinct and widely separated. Active hematopoiesis is noted throughout the spleen, and all stages of development of both granulocytic and erythrocytic cells are seen. Islands of these cells frequently fill the sinusoids or obscure the normal architecture, but there is no evidence of neoplastic invasion. Megakaryocytes are seen often in all areas. Plasma cells are not numerous, but can be found lying free in the sinusoids and in the splenic pulp. Numerous large histiocytes contain heavy deposits of golden brown pigment. These cells are seen lying free in the sinusoids, in the splenic pulp, and in Malpighian corpuscles.

Diagnosis.—Extramedullary hematopoiesis, congestion, and hemosiderosis. Sections of the liver were not remarkable. A touch preparation of the cut surface of the spleen

*Performed by Dr. W. Walker.

showed the following differential count (see Fig. 2, *D*): 1 eosinophil, 1 promyelocyte, 1 myelocyte, 1 metamyelocyte, 2 band forms, 12 segmented neutrophils, 63 small mature lymphocytes, 2 young lymphocytes, 1 reticulum cell, 1 phagocytic clasmatocyte, 15 plasma cells and 1 normoblast, 1 late erythroblast and 1 early erythroblast per 100 white cells.

The postoperative course was stormy with two episodes of shock. Re-exploration of the abdomen 24 hours postoperatively revealed diffuse bleeding from the pancreas. In addition, fever, transient left serosanguineous pleural effusion, and sinus tract formation in the splenectomy wound developed.

TABLE I. BONE MARROW FINDINGS AT INTERVALS IN A PATIENT WITH MACROGLOBULINEMIA

TYPE OF CELL	JULY, 1951 (PER CENT)	NOVEMBER, 1954 (PER CENT)	MARCH, 1955 (PER CENT)
Basophils			1
Eosinophils		5	
A Myelocytes			
B Myelocytes		1	
C Myelocytes		16	3
Metammyelocytes		18	3
Band forms		7	2
Segmented neutrophils	1.0	17	6
Lymphocytes	82.0	30	63
Plasma cells	15.5	4	21
Tissue basophils	1.5	Present	
Reticulum cells		1	
Megakaryocytes		1	
Normoblasts	1.0	29	16
Late erythroblasts		16	5
Early erythroblasts		1	
Total nucleated RBC (per 100 WBC)	1.0	46	21
Relative cellularity	Hypocellular	Hypercellular	Hypercellular

She was readmitted to Barnes Hospital for the fourth time on March 27, 1955, because of one week's duration of generalized abdominal pain, weakness, and epistaxis. Abnormal physical findings included debilitation, facial flush, pallor of the mucous membranes, a large ecchymosis over the left forearm, hepatomegaly extending 7 cm. below the right costal margin, exquisite tenderness over the entire abdomen, and the sinus tract in the splenectomy wound draining a small amount of clear fluid. The urinalysis revealed a trace of protein and 1 to 3 red blood cells per high power field. The total protein was 10.8 Gm. per cent, albumin 2.0 Gm. per cent, globulin 8.8 Gm. per cent and nonprotein nitrogen 35 mg. per cent. Lymphocytosis up to 66 per cent and normoblasts up to 4 per 100 white cells were again noted in the peripheral blood. Repeat sternal marrow examination revealed large clumps of cellular marrow (see Fig. 2, *C*), with a predominance of lymphocytes, increase in plasma cells and hemosiderin, depression of the myeloid cells, normal megakaryocytes and nucleated red cells, and many tissue basophils (Table I). Chest roentgenogram revealed elevation of the right diaphragm and discoid atelectasis of the right lower lung lobe. Injection of 30 per cent Urokon into the sinus tract revealed that it extended to the posterior left subphrenic region. The sinus tract fluid was found to contain 80,000 units amylase per milliliter.

Twenty-two hundred roentgens (in air) to the sinus tract resulted in almost complete cessation of drainage. Urethane therapy (38 Gm. over a 19-day period) was associated with a fall in total proteins of 1.5 Gm. per cent, due mainly to a reduction in the globulins.

INVESTIGATION OF THE ABNORMAL PROTEIN OF THE PATIENT

1. *Studies of the Plasma and Serum Proteins.*—

a. *Water (euglobulin) test:* A drop of the patient's plasma or serum in 10 ml. of distilled water quickly formed an intense white flocculent precipitate (temperature 25° C.).

The precipitate could be easily dissolved by the addition of saline and re-precipitated by the addition of distilled water. To determine the optimal pH requirement of the positive water test, we studied a 6 to 7 per cent preparation of the isolated macroglobulin, obtained by its precipitation and separation from plasma in distilled water, for maximum insolubility in distilled water at various pH levels. Maximum insolubility was observed at pH 6.40 with a sharp increase in solubility toward the more acid range and a more gradual increasing solubility toward the alkaline range. Random samples of distilled water stored in carboys in the laboratory ranged in pH from 6.00 to 6.70.

b. *Effect of cooling:* The plasma and serum kept at 4° C. for up to one week did not gel or precipitate. Cryoglobulin was considered to be absent.

c. *Electrophoresis:* Paper electrophoresis (Veronal buffer, pH 8.6, ionic strength 0.1) of the serum revealed a moderate decrease in albumin, slight decrease in alpha 2 and beta globulins, a small quantity of "normal" gamma globulin, and an abnormal, narrow concentrated band of slow gamma globulin mobility (Fig. 3, *a*). With the periodic acid-Schiff

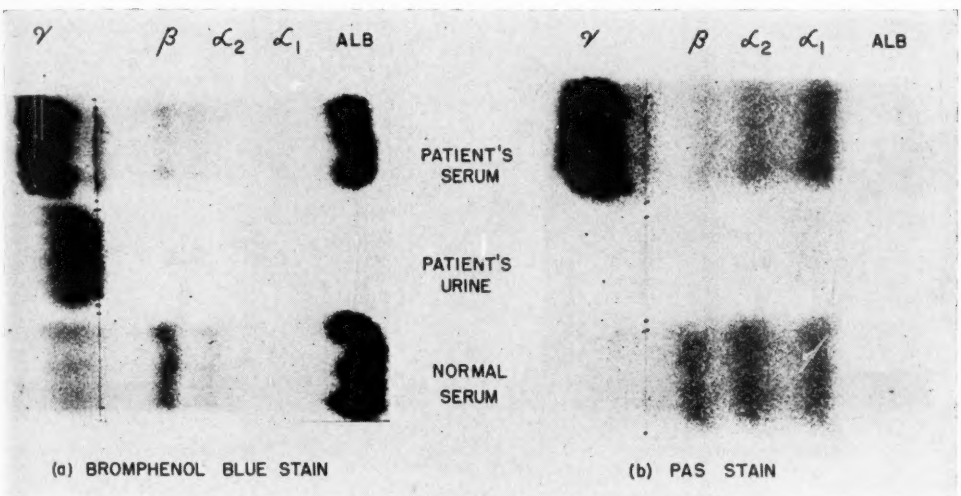

Fig. 3.—Paper electrophoresis of the patient's serum and urine.

(PAS) reagents, the abnormal gamma globulin band exhibited a markedly positive staining reaction for glyco-protein (Fig. 3, *b*). Tiselius electrophoresis of the serum* (Veronal buffer, pH 8.6, ionic strength 0.1, 180 minutes at 12 Ma. at 1° C. in a Spinco Model H electrophoresis diffusion apparatus) revealed the following mobilities and relative concentrations (Fig. 4) (Table II).

TABLE II

	MOBILITY* (X 10^{-5} CM.^{2}SEC.$^{-1}$ VOLT^{-1})	RELATIVE CONCENTRATIONS† (PER CENT)
Albumin	−6.1	22.1
Globulins		
Alpha 1	−5.2	4.0
Alpha 2	−3.8	6.6
Beta	−3.2	5.5
"Normal" gamma	−1.5	7.3
Macroglobulin	−0.5	55.0

*The values as reported by Dr. Putnam are only approximate and are subject to a final correction in the order of 5 per cent.

†Calculated by Carmelita Lowry, Department of Biochemistry, Washington University, St. Louis.

*Kindly performed by Dr. Frank W. Putnam, Argonne Cancer Research Hospital, Chicago, Ill.

d. *Ultracentrifugation studies:* The serum was diluted 1:10 with 0.9 per cent NaCl and centrifuged for 30 minutes in a Spinco Model E ultracentrifuge at 59,780 r.p.m.* The serum separated into four components with the following sedimentation constants and relative concentrations (Fig. 5):

3.9 S 40 per cent – albumin
6.6 S 7 per cent – globulins
13.9 S 48 per cent – macroglobulin
19.9 S 5 per cent – globulin
(S = Svedberg units)

The macroglobulin was obtained in pure form by prolonged dialysis against distilled water and found to sediment with three peaks in the analytic ultracentrifuge†; 6.1 S (15 per cent), 17 S (70 per cent), and 24 S (15 per cent).‡

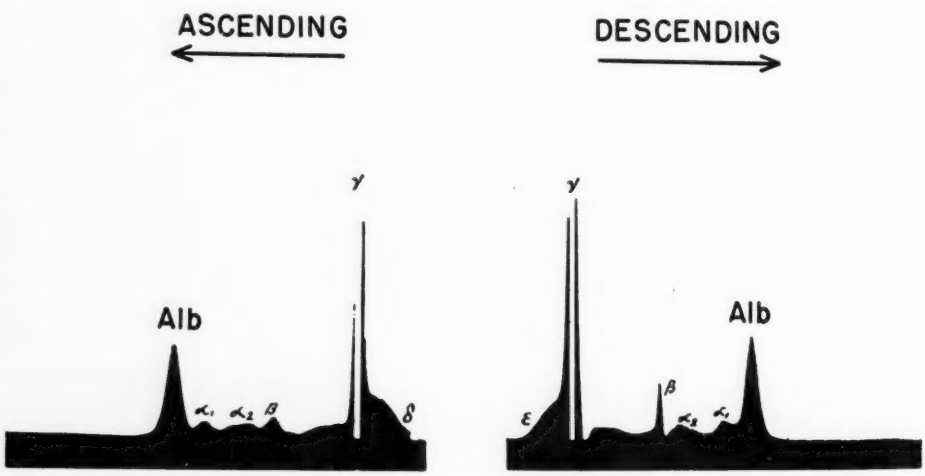

Fig. 4.—Electrophoresis (Tiselius) of the patient's serum. Note the narrow tall gamma globulin peak of slow mobility.

e. *Immunologic study§:* The patient's serum up to dilutions of 1:2,048 demonstrated a strongly positive reaction to antimacroglobin rabbit serum. Normal sera show weak reactions up to dilutions of 1:16. Immunologically, this serum behaved as a typical Waldenström's macroglobulinemia.

2. *Studies on the Urine.*—The routine heat test for Bence-Jones protein was negative, but the concentrated nitric acid ring test was positive for protein. One hundred and twenty milliliters of the urine was dialyzed for 20 hours against running water at 11° C. and concentrated to 2 ml. by evaporation. This concentrated urine gave a positive heat test for Bence-Jones protein. A drop of the concentrated urine did not precipitate in 10 ml. of distilled water. Paper electrophoresis of the concentrated urine revealed a homogeneous protein of intermediate gamma globulin mobility, slightly greater than its serum component (Fig. 3, *A*). The urinary protein did not stain with the PAS reagents (Fig. 3, *B*).

3. *Culture Studies of the Spleen for Microorganisms.*‖—Because a viral etiology for macroglobulinemia has been suggested,[7] culture studies were performed on the patient's

*Study done by Miss Lowry.

†Performed by Dr. F. W. Putnam.

‡Figures are approximate and are subject to a final correction in the order of 5 per cent.

§The immunologic study was very kindly performed and interpreted by Dr. A. Hässig, Serology Service of the Central Blood Transfusion Laboratory of the Swiss Red Cross, Berne, Switzerland.

‖Study done by Harold Ray, Bacteriology Diagnostic Laboratories, Barnes Hospital.

spleen. Twenty grams of the patient's spleen were homogenized in a Waring blendor and inoculated in fertile eggs. No bacterial or viral microorganisms were isolated by this technique from the spleen homogenate.

Study on other members of the family: Study of the total plasma proteins, water (euglobulin) test, and paper electrophoresis of the serum of seven other members of the family did not reveal macroglobulinemia.

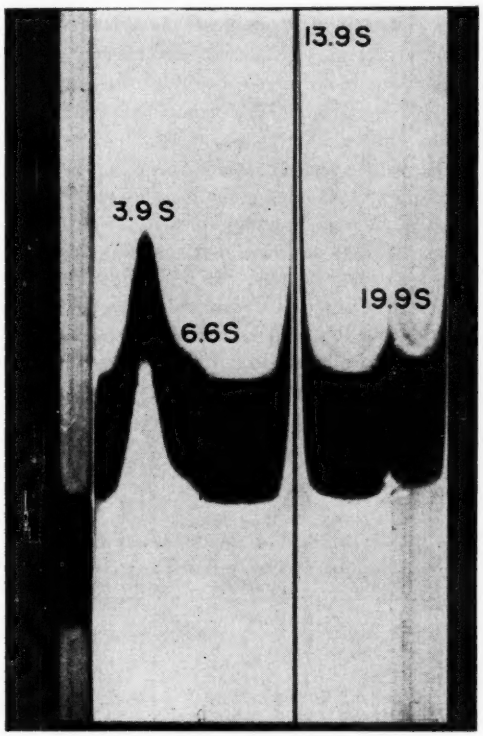

Fig. 5.—Ultracentrifugation pattern of the patient's serum. Note the large quantity of 13.9 S component (macroglobulin).

Investigation of the Patient's Coagulation Defect

Materials and Methods

All clotting procedures, except for the silicone tube clotting times, were carried out at 37° C. in 13 by 100 mm. glass tubes. Plasma was prepared by adding one part of 0.1 M sodium oxalate to 9 parts of whole blood and by separating the supernatant plasma by centrifugation at 2,500 r.p.m. for ten minutes. Plasma was defibrinated by successive additions of thrombin (50 units per milliliter) until no further fibrin was formed and was allowed to stand 45 minutes before use. Barium sulfate-treated plasma was prepared by incubating 1 ml. of normal oxalated plasma with 100 mg. powdered barium sulfate for ten minutes at 37° C., and the plasma was separated by centrifuging the mixture at 2,500 r.p.m. for 25 minutes at 4° C. (International Refrigerated Centrifuge). The following clotting agents were used: 0.025 M calcium chloride, thromboplastin (Permaplastin, C. W. Alban and Company), and a solution in saline of bovine thrombin (The Upjohn Company). Bovine fibrinogen was obtained from The Armour Laboratories.

Platelet counts were performed by the Dameshek method, capillary fragility by the Rumpel-Leede technique, and bleeding time by the Ivy method. Clotting times of the whole blood were determined in glass and siliconized tubes (15 by 125 mm. in size). Through an

18 gauge needle, after the discarding of the first few milliliters of blood, approximately 2 ml. of venous blood was allowed to flow directly into each of three glass tubes and 7 ml. into each of three siliconized tubes. Clot retraction was noted at two hours and an examination was made for fibrinolysins at 48 hours. The plasma prothrombin time was determined by the one-stage method of Quick. Serum prothrombin (one-stage) was estimated on 24-hour-old sterile serum with utilization of normal barium sulfate-treated plasma as a source of AC globulin and fibrinogen. AC globulin-poor plasma was prepared by incubation of normal oxalated plasma at 37° C. for 24 hours. Normal sterile serum was allowed to stand 24 hours before use. The time required for thrombin to clot plasma constituted the thrombin time. The fibrinogen concentration was determined by the method of Campbell and Hanna.[21] The macroglobulin was isolated by precipitation from plasma in an excess of distilled water and resuspended in saline in approximately 3 to 7 Gm. per cent concentration. By paper electrophoretic analysis, this suspension contained over 95 per cent homogeneous macroglobulin, a small amount of normal gamma globulin, and no fibrinogen. This material was not clottable by thrombin. A normal platelet suspension was prepared in the following manner: 20 ml. of normal whole blood was anticoagulated with 0.6 ml. of 5 per cent Sequestrene and centrifuged at 1,000 r.p.m. at 4° C. for 30 minutes, and the packed platelets were washed twice with an excess of cold saline and resuspended in 1 ml. of saline. The platelet suspension was frozen, thawed, and homogenized manually. The fibrinogen-like material in the platelet homogenate was clotted with thrombin and separated by centrifugation; the supernatant material was used.

RESULTS

The routine coagulation studies are listed on Table III.

TABLE III. ROUTINE COAGULATION STUDIES

	PATIENT	NORMAL VALUES
Platelet counts	See Fig. 1	300,000 to 900,000 per mm.^{3}
Rumpel-Leede test	Positive Negative*	Negative
Bleeding time	14½ minutes 2 to 3½ minutes*	Less than 7 minutes
Clotting time		
Glass tube	6 to 10 minutes*	Less than 15 minutes
Silicone tube	40 minutes 20 to 105 minutes*	Less than 120 minutes
Fibrinolysis	None observed at 48 hours	
Plasma prothrombin	20 to 100 per cent	100 per cent
Serum prothrombin	2 per cent	Less than 15 per cent
Fibrinogen	472 to 550 mg. per cent*	200 to 400 mg. per cent

*Postsplenectomy.

1. *Investigation of the Prolonged Prothrombin Time.*—

a. *Demonstration of AC globulin deficiency:* Studies on the 26th postoperative day revealed a failure of the patient's plasma to correct the prolonged prothrombin time of normal heated plasma (AC globulin poor); however, both normal and barium sulfate-treated plasma corrected to a large extent the patient's prolonged prothrombin time (Table IV). That normal barium sulfate-treated plasma (prothrombin-free and convertin-poor) provided as much correction as normal plasma excluded any significant prothrombin or convertin deficiency at that time. Furthermore, normal serum failed to correct the patient's delayed prothrombin time. The isolated macroglobulin did not interfere with the ability of normal barium sulfate-treated plasma (containing AC

globulin) to correct both the prolonged prothrombin times of normal heated plasma and of the patient's plasma. The failure of normal and barium sulfate-treated plasmas to restore completely the prothrombin time of the patient suggested additional coagulation defects.

TABLE IV. DEMONSTRATION OF AC GLOBULIN DEFICIENCY

PATIENT'S PLASMA (ML.)	NORMAL PLASMA (ML.)	NORMAL BARIUM SULFATE PLASMA (ML.)	AC GLOBULIN-POOR PLASMA (ML.)	SALINE (ML.)	CLOTTING TIME (SEC.)
			0.1	0.05	54.8
0.05			0.1		53.0
	0.05		0.1		15.2
0.1	0.05				16.9
0.1		0.05			16.0
0.1				0.05	21.7
	0.1			0.1	12.3

One-tenth milliliter of thromboplastin and 0.1 ml. of calcium chloride were added to the above mixtures and the clotting times were determined.

b. *Demonstration of prothrombin deficiency:* On the 74th postoperative day, slight AC globulin deficiency was still present, but moderate hypoprothrombinemia was now evident. The patient's plasma almost completely corrected the clotting time of normal heated plasma; however, normal barium sulfate-treated plasma (deficient in prothrombin and convertin) did not correct the patient's prolonged prothrombin time as well as did normal plasma (Table V). The addition of normal serum to the patient's plasma did not result in significant improvement in the prolonged prothrombin time.

TABLE V. DEMONSTRATION OF PROTHROMBIN DEFICIENCY

PATIENT'S PLASMA (ML.)	NORMAL PLASMA (ML.)	AC GLOBULIN-POOR PLASMA (ML.)	NORMAL BARIUM SULFATE-TREATED PLASMA (ML.)	NORMAL SERUM (ML.)	SALINE (ML.)	CLOTTING TIME (SEC.)
0.1						22.3
	0.1					12.0
		0.1			0.1	42.1
0.1		0.1				15.1
	0.1	0.1				14.5
0.1			0.1			30.0
0.1	0.1					14.0
0.1				0.1		19.2
	0.1			0.1		8.5

One-tenth milliliter of thromboplastin and 0.1 ml. of calcium chloride were added to the above plasma mixtures and the clotting times were determined.

2. *Absence of Antithromboplastin.*—Serial dilutions of thromboplastin with saline to 1:5,000 failed to disclose significant prolongation of the patient's prothrombin time over that of the normal control.

3. *Presence of Antihemophilic Globulin (AHG).*—Equal parts of the patient's plasma and that of a known hemophiliac were recalcified and the serum prothrombin time performed on the serum of the mixture 24 hours later. The addition of the patient's plasma resulted in normalization of the poor prothrombin consumption of the hemophiliac plasma.

4. *Demonstration of Prolonged Thrombin Time.*—The addition of thrombin in different concentrations consistently resulted in prolonged thrombin times (Fig. 6).

5. *Decreased Antithrombin.*—Bovine thrombin incubated with the patient's 24-hour-old serum (Fig. 7) and with the patient's defibrinated plasma (Table VI) resulted in more rapid clotting of the mixture upon addition of bovine fibrinogen than similarly treated controls using normal 24 hours' serum and defibrinated plasma, suggesting a decrease in antithrombin content of the patient's blood.

TABLE VI. DECREASE IN ANTITHROMBIN

	CLOTTING TIME (SEC.)
Patient's defibrinated plasma	19.3
Normal defibrinated plasma	38.2

One-tenth milliliter of thrombin (25 μ/ml.) was incubated for 2 minutes in 0.1 ml. of the patients's defibrinated plasma and in 0.1 ml. of normal defibrinated plasma. One-tenth milliliter of bovine fibrogen (600 mg. %) was added to the incubated mixture and the clotting time was noted.

6. *Lack of Interference of the Isolated Macroglobulin on the Clotting Effect of Thrombin on Normal Plasma.*—The isolated macroglobulin did not inhibit the clotting action of thrombin on normal plasma.

7. *Failure of the Patient's Plasma and the Isolated Macroglobulin to Block the Conversion of Fibrinogen to Fibrin.*—The patient's defibrinated plasma as well as the isolated macroglobulin did not interfere with the conversion of bovine fibrinogen to fibrin by thrombin.

8. *Lack of Effect of Calcium Ions on the Prolonged Thrombin Time.*—The addition of calcium chloride to the patient's plasma did not shorten the prolonged thrombin time but accelerated the thrombin time of the normal control plasma. The accelerating effect of calcium on the thrombin time of normal plasma has been previously described.[22]

9. *Lack of Deficiency of the "Fibrinogen Conversion Accelerator Factor."*—The addition of fresh defibrinated plasma or normal serum to the patient's plasma did not result in shortening of the patient's prolonged thrombin time (Table VII). However, normal plasma (containing fibrinogen) improved the prolonged thrombin time of the patient. A deficiency of the "fibrinogen conversion accelerator factor"[23] was not believed to be present.

TABLE VII. LACK OF DEFICIENCY OF THE "FIBRINOGEN CONVERSION ACCELERATOR FACTOR"

PATIENT'S PLASMA (ML.)	NORMAL PLASMA (ML.)	DEFIBRINATED NORMAL PLASMA (ML.)	SALINE (ML.)	CLOTTING TIME (SEC.)
0.4			0.1	27.0
	0.4		0.1	14.1
0.4	0.1			19.2
0.4	0.3			24.0
0.4		0.1		36.0
0.4		0.1*		27.0
0.4		0.3		118.0

One-tenth milliliter of thrombin (10 μ/ml.) was added to the above plasma mixtures and the clotting times were determined.

*Whole blood defibrinated with glass beads.

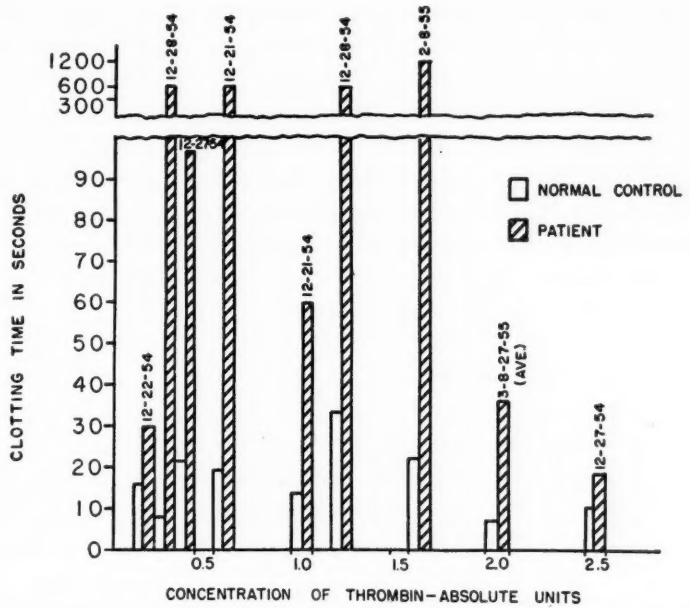

Fig. 6.—Demonstration of the patient's prolonged thrombin times. 0.1 to 0.2 ml. of thrombin (concentration expressed as absolute units of thrombin added) was added to 0.1 ml. of the patient's plasma and 0.1 ml. of normal plasma (control), and the clotting times (seconds) were determined. The use of different lots of thrombin dissolved in saline or glycerol water resulted in slight variation of the normal control thrombin times.

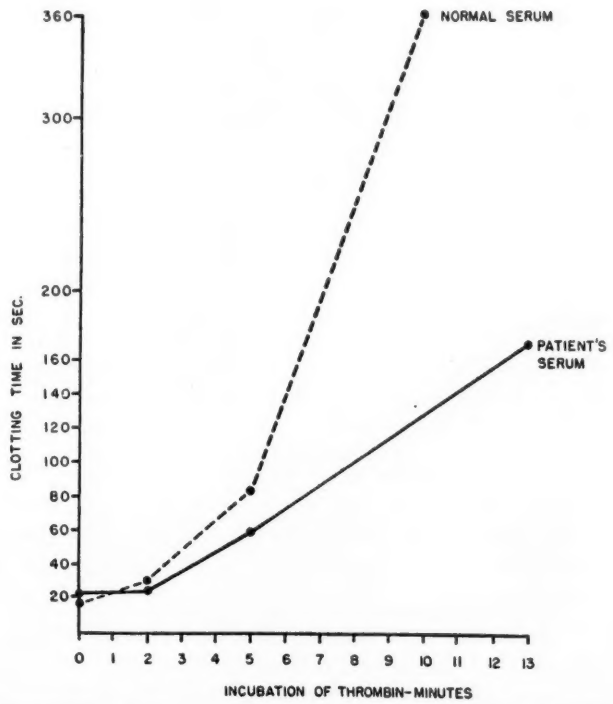

Fig. 7.—Demonstration of decreased antithrombin. 0.1 ml. of thrombin (2 μ/ml.) was incubated in 0.1 ml. of the patient's serum and in 0.1 ml. of normal serum. 0.1 ml. of bovine fibrinogen (600 mg. %) was then added to the incubated mixture and the clotting time determined (seconds).

10. *Effect of Protamine Sulfate on the Thrombin Time.*—The addition of protamine sulfate (in concentrations varying from 0.01 to 1.0 mg. per milliliter) to the patient's plasma did not produce significant changes in the thrombin time over that of the normal control. An excess heparinemia was therefore not believed to be responsible for the patient's prolonged thrombin time.

11. *Lack of Effect of a Normal Platelet Suspension on the Patient's Prolonged Thrombin Time.*—To 0.4 ml. of the patient's plasma was added 0.1 ml. of a normal platelet suspension and 0.1 ml. of thrombin (10 units per milliliter). The addition of the normal platelet suspension, containing a small amount of thrombin, did not shorten the prolonged thrombin time of the patient.

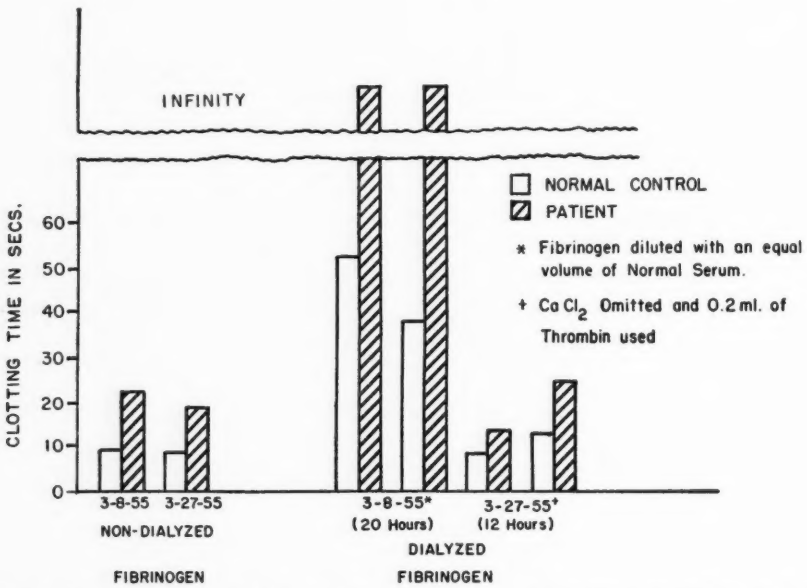

Fig. 8.—Demonstration of a qualitative defect in the clotting reactivity of the patient's isolated fibrinogen. 0.1 ml. of calcium chloride and 0.1 ml. of thrombin (10 μ/ml.) were added to 0.1 ml. of the patient's isolated fibrinogen and to 0.1 ml. of a normal isolated fibrinogen and the clotting times (seconds) determined prior to and following dialysis of the isolated fibrinogen.

12. *Failure of the Isolated Macroglobulin to Alter the Clotting Reactivity of Fibrinogen.*—Incubation of bovine fibrinogen up to 40 minutes with the isolated macroglobulin did not result in loss of clotting reactivity of the bovine fibrinogen to thrombin.

13. *Demonstration of a Qualitative Defect in the Reactivity of the Patient's Fibrinogen to Clotting.*—Fibrinogen was isolated from the patient's plasma by precipitation with phosphate buffer by the method of Jaques[24] and dialyzed against running water at 11° C. Electrophoretic studies showed that this material contained only fibrinogen. Fibrinogen obtained from a normal subject with a fibrinogen concentration of 425 mg. per cent was prepared in the same manner. The addition of thrombin and calcium chloride to the patient's nondialyzed isolated fibrinogen resulted in a clotting time much longer than the normal fibrinogen (Fig. 8). The fibrinogen of the patient dialyzed for 20

hours failed to clot, while the normal control fibrinogen clotted in approximately 52 seconds. Thrombin and calcium chloride added to the dialyzed fibrinogen diluted with an equal volume of normal serum failed to clot the patient's fibrinogen, whereas the normal fibrinogen clotted in 38 seconds. Dialysis of the isolated fibrinogen for only 12 hours again revealed significant prolongation of the patient's clotting time over that of the normal control. The deficient clotting response of the patient's fibrinogen to thrombin suggested a qualitative defect in the fibrinogen itself and appeared to be the essential defect in the consistently prolonged thrombin times of the patient.

DISCUSSION

Manifestations of Macroglobulinemia.—In 1944 Waldenström first described a disease entity which he later named "macroglobulinemia" and which has been recorded by others.[1-20] Primary macroglobulinemia has been reported most often in elderly males. The symptomatology is vague and of many years' duration with insidious onset of weakness, asthenia, lassitude, dyspnea, weight loss, occasional fever, and central nervous system degeneration. Hemorrhagic tendencies are prominent and may consist of purpura, retinal hemorrhages, epistaxis, bleeding gums, and hemorrhages into the central nervous system. Bone pain is conspicuously absent. The course is usually chronic but may be rapidly downhill. Physical examination may show, in addition to hemorrhagic manifestations, marked pallor, vascular disturbances of the extremities, variably enlarged nontender lymph nodes, hepatosplenomegaly and edema.

The urine may contain Bence-Jones protein. The anemia is usually normochromic, moderately severe, and persistent. It infreqently is hemolytic in nature. There may be moderate leukopenia, normal white cell count, or slight leukocytosis with nonspecific findings as eosinophilia, lymphocytosis, or monocytosis. Severe thrombocytopenia may develop. A high sedimentation rate is almost uniformly found. The hyperproteinemia is primarily a hyperglobulinemia associated with hypoalbuminemia. Infrequently cryoglobulins are noted. A drop of the serum falling into distilled water forms an intense flocculent white precipitate which dissolves on the addition of saline and reprecipitates on the addition of distilled water.

Roentgen examination is not revealing except for slight generalized decalcification of the bones. The typical punched-out bone lesions of myeloma are not seen.

Treatment has been uniformly ineffective. Urethane, nitrogen mustard, radioactive phosphorus, and cortisone have not affected the basic disease process. Amidin derivative 2834-37 (2 methoxy-phenoxy-aceto-N,N-pentamethyleneamidine-hydrochloride) administration to one patient was associated with reduction of lymphocytic bone marrow infiltrate and to another patient with reversion of a positive water test to a negative one.[13] In the patient here reported, cortisone and splenectomy did not influence the basic disease process.

Etiology.—The etiology of primary macroglobulinemia is unknown. Waldenström[7] suggested it is due to a virus but isolation of specific organisms have not been reported. Culture of extracts of the spleen of the present case revealed no bacterial or viral organisms. Excess production of large molecular weight globulins appears to be the basic defect in macroglobulinemia. The precise site of origin and whether they present abnormal or normal protein molecules in abnormally large amounts are not known. That the macroglobulin is of cellular origin, whether reticuloendothelial, plasmocytic or lymphocytic, is suggested by many clinical observations. "Paraproteins"* have been reported in association with myeloma,[7, 25] leukemia,[26] lymphosarcoma,[27] and generalized carcinomatosis.[7] In the myeloma cell the large dark droplike cytoplasmic granulations observed under the phase contrast microscope[28] are thought to represent abnormal protein formation. Electrophoretic patterns of extracts of lymph nodes and of ileum infiltrated with myeloma cells showed identical large, sharp, tall peaks, as were seen in the plasma electrophoresis of a patient with myeloma.[29] More recently, large amounts of intracellular periodic-acid Schiff (PAS) positive material, probably protein in nature, have been described in malignant lymphoma cells, including reticulum cell sarcoma, lymphosarcoma, and chronic lymphocytic leukemia.[30] In myeloma the concentration of the abnormal plasma proteins usually, but not always, parallels the number of plasma cells in the bone marrow,[31] while the degree of plasmocytosis appears less striking than the amount of abnormal protein present in macroglobulinemia.

Histopathology.—Varying degrees of reticuloendothelial proliferation, plasmocytosis, or lymphocytic hyperplasia may be found by bone marrow aspiration or at autopsy in macroglobulinemia. Mast cell hyperplasia of the bone marrow and lymph nodes has been reported.[4] The plasmocytosis may be slight or moderate in degree, mature in nature, or more pleomorphic with atypical appearance, but without the diffuse or nodular involvement of myeloma cells seen in myelomatosis. The lymphocytic proliferation is often indistinguishable from lymphocytic leukemia, with lymphoid cells often comprising 70 to 90 per cent of the bone marrow.[14] Occasionally, however, the marrow may be hypocellular[13] or normal.[20]

In the patient reported, no definite diagnosis could be made on examination of the marrow aspirates or preparations of the spleen. In Table I are presented the three sternal marrow differential counts obtained. There is a striking difference between that obtained in November, 1954, and on the other two examinations. The apparent change in the bone marrow cytology may have been caused by focal areas of involvement or different stages of the disease process. This changing bone marrow pattern may account for the variable and inconstant histologic picture reported in macroglobulinemia. The histologic nature of the plasma cells in all preparations was the same and varied from the normal mature plasma cell to immature atypical cells similar to those found in instances of myeloma.

*Paraproteins refer to abnormal plasma proteins which are electrophoretically characterized by tall, narrow, sharp peaks, indicative of marked homogeneity.

However, because of the marrow plasmocytosis and lymphocytosis in the peripheral blood and bone marrow, the possibility of myeloma or lymphocytic leukemia could not be completely excluded.

Physico-Chemical Considerations.—Despite many similarities, the macroglobulinemia protein differs from the myeloma protein and cryoglobulin in many respects. By electrophoretic analysis, all three usually exhibit narrow, tall, sharp peaks characteristic of protein homogeneity, but on rare occasions less tall, broader peaks have been observed in myeloma,[32] and macroglobulinemia.[13] All migrate most often with mobilities similar to that of gamma globulin, less frequently to that of "M" globulin, least often to that of beta globulin, and occasionally in myeloma to that of alpha globulin.[7] Occasionally more than one abnormal homogeneous component[33, 34] and as many as four distinct peaks within a single abnormal electrophoretic homogeneous component of gamma globulin mobility may be seen in the myeloma sera.[35]

By ultracentrifugation study, differences between the macroglobulinemia and myeloma proteins are found. In myeloma the sedimentation constant of the serum usually ranges from 6.1 to 8.3 S, with smaller amounts of more rapid sedimenting components often being present.[35] The sedimentation constants of the isolated myeloma protein are usually 6 to 7 S[33, 36, 37] and less often 9 to 9.5 S.[36, 37] However, myeloma proteins with higher sedimentation constants of 15.4 and 17.7 S have been reported.[17, 38] The isolated myeloma proteins, like whole sera, may also demonstrate smaller amounts of more rapid sedimenting components in addition to the main 6 to 7 S or 9 to 9.5 S components. The macroglobulinemia sera in general demonstrate consistently high sedimentation constants, usually in the 19 to 20 S range. Study of the purified macroglobulin fractions in the ultracentrifuge tends to reveal small amounts of slow or faster sedimentation constants in addition to the main component.

The molecular weight of most of the myeloma proteins is approximately 120,000 to 300,000,[33, 36] while the macroglobulin proteins are approximately 1,000,000.[7] However, the evidence would indicate the occasional formation of larger molecular weight proteins in myeloma. In a myeloma patient with an isolated protein sedimentation constant of 9.5 S, the molecular weight was approximately 400,000.[36] In the myeloma patients with sedimentation constants of 15.4 S and 17.7 S,[17, 38] molecular weight determinations were not performed, but the abnormal proteins were most likely of large molecular weight. Although molecular weight determination was not done on the patient reported, the purified protein sedimentation constant of 17 S would indicate a globulin of sizeable molecular weight, probably near 1,000,000.[39]

Cryoglobulins are abnormal plasma proteins that spontaneously gel or precipitate at lower temperature and redissolve on warming. In whole sera or in its isolated state, they have sedimentation constants whose main components range between 6 and 20 S and often minor components sedimenting more rapidly between 20 and 30 S.[6, 16, 17, 27, 41, 43-47] The molecular weights of cryoglobulins have ranged from 100,000 to 1,800,000.[4, 27, 40-45] Probably closely

related to the cryoglobulins are the spontaneous cold precipitable proteins that do not redissolve on warming, observed in periarteritis nodosa, cirrhoses, and subacute bacterial endocarditis.[48]

The sera of patients with myeloma and macroglobulinemia occasionally demonstrate the properties of cryoglobulins.[3, 4, 6, 17, 44] Relatively lower molecular weight cryoglobulins are found in myeloma (100,000 to 200,000),[40, 41] cardiovascular-renal disease and acropurpura (190,000),[42] lymphosarcoma (600,000),[27] and myelosclerosis (between 400,000 and 1,000,000).[43*] In contradistinction, the cryoglobulins found in macroglobulinemia are of higher molecular weight, i.e., 1,080,000[1] and 1,800,000.[4] Other cryoglobulinemias of high molecular weight appear to resemble closely or are probably variants of Waldenström's primary macroglobulinemia. The patient reported by McFarlane and associates,[45] with a cryoglobulin of approximately 1,000,000 molecular weight had clinical and necropsy changes similar to those found in patients with macroglobulinemia. In a patient reported by Gard and associates,[44] with clinical features resembling macroglobulinemia the abnormal protein was a cryoglobulin with an estimated molecular weight of 1,000,000. More recently Petermann and co-workers[46] have described a cryoglobulin with high sedimentation constants presumably of high molecular weight in a patient who did not present the typical findings of myeloma at autopsy.

The occasional association of macroglobulinemia in a number of diseases including nephrosis,[7] lupus erythematosus,[7] congenital syphilis,[15] Sjögren's syndrome,[8] extramedullary plasmocytoma,[10] myeloma,[17] and carcinoma of the uterus[9] would suggest its nonspecific formation in a variety of disease states (secondary macroglobulinemia).

The amino acid compositions of the macroglobulin,[5, 14, 16] cryoglobulin,[50] and myeloma proteins[38] in general show close similarities to normal gamma globulin. The macroglobulin may present a pattern lying between normal gamma and beta globulin, often more closely resembling gamma or beta globulin.[14] Examination of the individual amino acids in macroglobulinemia fails to disclose any constant specific pattern or alteration. The amino acid composition of the Bence-Jones proteins also does not show marked structural differences[5, 51-53] but has less similarity to normal gamma globulin than to macroglobulins.

In the case reported the macroglobulin demonstrated by paper electrophoresis revealed a positive PAS reaction similar to that observed with myeloma proteins[54] and cryoglobulins.[55] The positive PAS reaction exhibited by the myeloma, cryoglobulin, and macroglobulin paraproteins would suggest a closely related glycoprotein defect.

Many of the macroglobulins are euglobulins and are insoluble in electrolyte-free water. An optimal pH also appears important. Proteins are in general least soluble at their isoelectric points. In the present case the isoelectric point

*Final autopsy pathologic diagnosis.[49]

of the isolated macroglobulin in distilled water was at a pH (6.40), close to that of nonfreshly distilled water (6.00 to 6.70). The macroglobulin reported by Kratochvil[19] also had a similar isoelectric point (pH 6.35).

The water test, while not diagnostic in itself, appears to be a useful screening test for the presence of macroglobulinemia. It is occasionally negative in the presence of large as well as small amounts of macroglobulins.[7] The water test was markedly positive in the patient reported. In 378 other sera tested in this laboratory the water test was found to be negative or only slightly turbid. Included were sera from 17 normal individuals, 28 myeloma patients, 36 patients with acute and chronic leukemia, 16 patients with malignant lymphoma, 5 patients with cancer, plus 276 patients with miscellaneous diseases. Two patients with cryoglobulinemia were observed. In one, a woman with periarteritis nodosa, the water test was negative. The second patient was a man in whom the clinical diagnosis has not definitely been established. His serum gave a positive water test; the precipitate dissolved in saline but did not reprecipitate upon the addition of distilled water. The precipitate may have been a false positive reaction in which the cryoglobulin precipitated because of the cooling effect of the water itself. When the test was repeated at 50° C., no precipitate formed, whereas it appeared under similar conditions with the serum of the patient with macroglobulinemia on whom this report is based.

Bence-Jones proteinuria may be occasionally found in macroglobulinemia.[12, 14, 16] The Bence-Jones proteinuria in this case report shows similar characteristics to the Bence-Jones protein in myeloma.[54] The electrophoretic homogeneity and more rapid mobility than its serum component and failure to stain with the PAS reagent characterize many of the myeloma Bence-Jones proteins.

Immunology.—Specific rabbit immune antisera produced against the macroglobulins provide immunologic evidence for their antigenic specificity. Habich[18] found that all of 16 macroglobulin sera reacted generally with the prepared rabbit immune antisera as a group and also that the individual macroglobulins reacted specifically to their own homologous antiserum. This result suggested different antigenic specificities among the macroglobulins. Antigenic differences between the macroglobulin and myeloma proteins were also demonstrated by Habich; the sera from 11 patients with plasmocytoma failed to react with the macroglobulin rabbit immune antisera.

Hemostatic Defects.—A variety of coagulation defects involving the vascular, platelet, and plasma clotting factors may be found in macroglobulinemia and the related paraproteins. In myeloma, delayed glass, and silicone clotting times, slightly prolonged prothrombin times, prolonged thrombin times, deficiency of the "fibrinogen conversion accelerator factor," and normal or elevated fibrinogen concentrations have been reported.[56] Studies have also indicated specific interference of the conversion of fibrinogen to fibrin by the abnormal myeloma protein.[57-59] In macroglobulinemia, defects consisting of

prolonged bleeding, clotting, and prothrombin times, decreased AC globulin and convertin, poor prothrombin consumption, but low to normal fibrinogen concentrations have been found.[1-4, 10, 11, 15]

In the case reported, the positive Rumpel-Leede test and prolonged bleeding time were probably due to the thrombocytopenia. With administration of cortisone and after splenectomy, the Rumpel-Leede test and bleeding time reverted to normal with a rise in platelet count to normal. The thrombocytopenia was not due to myelophthisic replacement of the marrow megakaryocytes, as these cells were plentiful in the bone marrow despite the lymphocytic and plasmocytic infiltration. The splenomegaly, adequate numbers of marrow megakaryocytes, and platelet response to cortisone and splenectomy all suggest dyssplenism as the main factor causing the thrombocytopenia.

Abnormalities in some of the plasma clotting factors were also evident. The prothrombin time was prolonged, the AC globulin and prothrombin were decreased at various times, thrombin time was delayed, and antithrombin was decreased. An inhibitory or antagonistic effect of the macroglobulin upon the various phases of the patient's coagulation mechanism as studied in vitro could not be demonstrated. The essential defect, however, appeared to be one of a qualitative abnormality in the fibrinogen itself with an impaired response to clotting.

SUMMARY

The clinical case report of a 79-year-old white woman with macroglobulinemia is presented. Pallor, hepatosplenomegaly, and hemorrhagic manifestations were noted with Bence-Jones proteinuria, anemia, thrombocytopenia, and bone marrow plasmocytosis and lymphocytosis. Various coagulation defects were found, including reduction in AC globulin and prothrombin, prolongation of the thrombin time, and an impaired clotting response of the fibrinogen to thrombin. The macroglobulin was characterized by a sedimentation constant of 17 S, a narrow, tall, sharp electrophoretic peak of relatively slow gamma globulin mobility, and a markedly positive PAS staining reaction on paper electrophoresis. An abnormal protein electrophoretically homogeneous and of greater mobility than its serum component was also found in the urine. Splenectomy failed to alter the basic disease process but resulted in correction of the thrombocytopenia. Culture studies of the removed spleen failed to reveal microorganisms. Although the basic disease process in the patient reported could not be established unequivocally and the possibility of myeloma or lymphocytic leukemia cannot be entirely excluded, the disease process appears to resemble most closely that of Waldenström's primary macroglobulinemia. The relationship of the macroglobulinemia to the other paraproteinemias is also discussed.

CONCLUSIONS

1. Macroglobulinemia appears to be produced in a variety of disease states. Primary macroglobulinemia (Waldenström) appears to be a distinct entity,

with multiple hematologic and related clinical abnormalities. Secondary macroglobulinemia is occasionally observed as a nonspecific response in a variety of diseases.

2. The basic defect in primary macroglobulinemia appears to be a primary disturbance of the reticuloendothelial system with an abnormal production of high molecular weight globulin.

3. Primary macroglobulinemia resembles but is probably a separate disease entity from myeloma.

4. There is a variety of paraproteinemias, of which macroglobulinemia is one, which differ on the basis of their physicochemical, biochemic, and immunologic properties.

We wish to acknowledge the many suggestions and helpful guidance of Dr. William J. Harrington throughout this study and to thank him and Dr. Carl V. Moore for their careful review of the manuscript.

ADDENDUM

The subsequent course of the patient after the last hospital discharge was uneventful until Nov. 7, 1955. At this time she developed signs of acute infection with fever, chills, and lumbar pain, and she soon became comatose. Blood studies showed: hemoglobin 9 Gm. per cent, R.B.C. 3,070,000 per mm.^{3}, W.B.C. 10,250 per mm.^{3}, segmented neutrophils 48 per cent, stabs 25 per cent, metamyelocytes 1 per cent, and lymphocytes 24 per cent. Serum amylase was 400 Somogyi units. Friedländer's bacillus was cultured from the blood. Death occurred within 12 hours after the onset of the terminal event. No autopsy was obtained.

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THE ACTION OF SIX ANTIBIOTICS SINGLY AND IN COMBINATION ON ENTEROCOCCI ISOLATED FROM CASES OF SUBACUTE BACTERIAL ENDOCARDITIS

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MOST cases of subacute bacterial endocarditis are caused by penicillin-sensitive streptococci, and their treatment with this drug is generally successful.[1] In 5 to 14 per cent of recorded cases, however, the organism is an enterococcus which is relatively resistant to penicillin,[2-4] and treatment may present great difficulties. Clinical reports indicate that chloramphenicol, chlortetracycline, and oxytetracycline are not effective drugs, despite the fact that the organisms may appear to be highly sensitive to them in vitro.[5, 6] The best results have been obtained by the maintenance of high blood levels of penicillin,[7, 8] or by use of a combination of penicillin and either streptomycin or bacitracin.[9, 10, 11] Chloramphenicol, chlortetracycline, and oxytetracycline possess a predominantly bacteriostatic action, whereas penicillin and streptomycin are bactericidal. Hunter[12] suggested that for the cure of subacute endocarditis, bactericidal action is required and that inhibition of growth is not sufficient. The success of combinations of drugs can probably be attributed to increased bactericidal action, an effect which can be demonstrated in vitro when certain pairs of drugs are used together.[13, 14]

A full laboratory investigation of organisms isolated from cases of subacute endocarditis should, therefore, include not only a study of the inhibitory concentration of various antibiotics but also their bactericidal effect when used alone or, if necessary, in combination. A method recently described was designed for this purpose and can be performed easily in a routine laboratory.[15] With its aid we have tested seven antibiotics, separately and combined in pairs, for their lethal action against enterococci, isolated from cases of endocarditis and refractory to penicillin treatment.

MATERIALS AND METHODS

Eighteen strains of streptococci isolated from cases of subacute endocarditis were tested. Fifteen were typical enterococci, belonging to Lancefield's Group D; the rest could not be grouped, but since they grew in the presence of bile salt, they were also classed as enterococci. The seven antibiotics used were penicillin, streptomycin, erythromycin, bacitracin, chloramphenicol, chlortetracycline, and oxytetracycline; a standard nutrient medium was used throughout.[16]

Bacteriostatic Action.—This was tested by the tube method with use of doubling dilutions of the drugs in glucose broth. The "sensitivity" of the organism was expressed as the minimum concentration of the drug capable of inhibiting visible growth.

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Bactericidal Action.—The basis of the method was the transfer of the organisms from the surface of one agar plate containing antibiotic (called the "primary" plate) to the surface of a second plate free of drug (the "replica" plate), by means of a velvet stamp.[17] Each organism was inoculated into glucose broth and incubated at 37° C. for about 4 hours until definite turbidity appeared. Loopfuls were placed on the surfaces of agar plates containing various concentrations of each of the antibiotics; the steps chosen ranged from the bacteriostatic concentrations up to the blood levels, obtainable therapeutically. Chloramphenicol was tested, however, also at much higher concentrations. Each loopful was spread into a circle approximately 1 cm. in diameter; several organisms could thus be tested on a single plate. The plates were incubated for 24 hours, and replica plates then made. One per cent glucose was added to the base medium of the replica plates for obtainment of

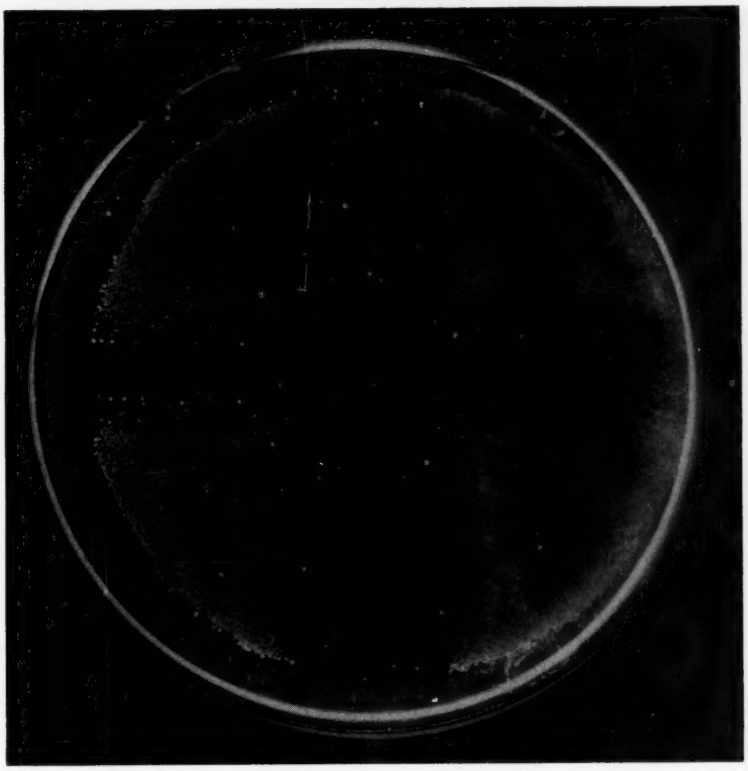

Fig. 1.—Replica plate illustrating the bactericidal effect of streptomycin (vertical) and chlortetracycline (horizontal) against an enterococcus. The square corners of the zones of growth indicate that there has been no interaction between the two antibiotics.

larger colonies and rendering of easier reading. The degree of killing was estimated by performance of the following experiment: tenfold dilutions of an inoculum were plated out, with use of loopfuls as already described, on two plates; replica plates were then made from one of them without intermediate incubation, while the second primary plate was incubated as control. In this way it was found that the method detected 99 per cent killing with the inoculum size described. When compared with the tube method described by Chabbert,[18] the replica method was found to be approximately 1/10 as sensitive. However, by use of a heavier original inoculum, such as a loopful of a 24-hour culture, the sensitivity can be increased 10- or 100-fold. For consistent results, the density of the original inoculum should be controlled turbidimetrically.

Interaction Effects.—The method was described in detail elsewhere.[15, 19] Briefly, it consisted of uniform inoculation of the surface of a nutrient agar plate with a 4 hours' culture in glucose-broth. Blotting-paper strips (6 mm. by 60 mm.) soaked in the individual antibiotics were placed at right angles to each other in the form of a cross. The concentrations were chosen to give inhibition zones of from 2 to 3 cm. in width; these were penicillin, 50,000 units/ml.; streptomycin, 10,000 mcg./ml.; chloramphenicol, 20,000 mcg./ml.; chlortetracycline and oxytetracyline, 12,500 mcg./ml.; bacitracin, 5,000 mcg./ml.; erythromycin, 500 mcg./ml. It has been found that a mistaken impression of bactericidal action may be formed if the zone size is not within these limits. If the inhibition zones are larger, the bactericidal action

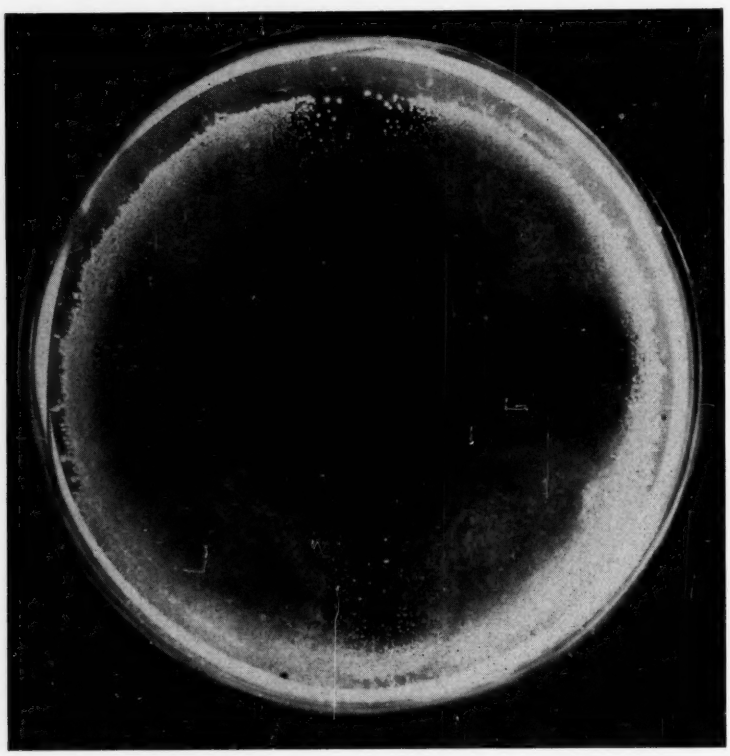

Fig. 2.—Replica plate illustrating the bactericidal effect of erythromycin (horizontal) + chlortetracycline (vertical). There is marked rounding of the corners of the zones of growth showing a synergistic action where the 2 drugs met.

of the antibiotic may appear greater than is actually the case; if smaller, the zone may be lost for technical reasons. Replica plates, made after 24 hours' incubation, could be interpreted as showing no interaction of the drugs (Fig. 1) or synergism (Fig. 2), demonstrated by the fact that the bactericidal action of the two drugs was greater than that of either alone, or antagonism (Figs. 3 and 4), in which both drugs, acting together, allowed more bacterial growth than one acting alone.

With use of this diffusion method, a range of concentrations is obtained in the agar extending from the edges of the strips, where it is highest to the point furthest away, where the concentration is virtually nil. Irrespective of the high concentrations for impregnating the paper strip, the edge of inhibition always represents the same concentration, i.e., that which is inhibitory for the particular organism.

EXPERIMENTAL FINDINGS

The minimum inhibitory concentrations of the seven antibiotics for the eighteen strains of enterococci are shown in Table I. The resistance to penicillin and streptomycin is evident and it was primarily because of this characteristic that the organisms were chosen for this investigation. Table II illustrates the bactericidal activity of all the various drugs. The results are in complete accord with the generalization that penicillin and streptomycin are bactericidal at levels not much greater than the inhibitory concentrations, while chloramphenicol, chlortetracycline, and oxytetracycline fail to kill in concentrations many

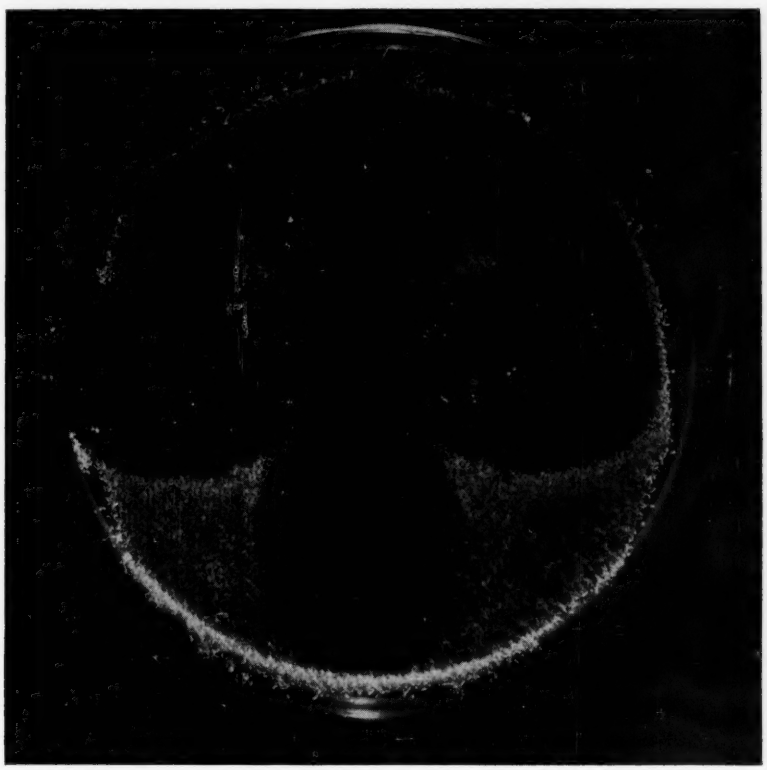

Fig. 3.—Replica plate illustrating the bactericidal effect of penicillin (horizontal) + bacitracin (vertical). Antagonism between the 2 drugs is shown by the tongues of growth occurring in the zone where antagonism between two bactericidal drugs is unusual.

times greater than their inhibitory levels. Bacitracin is bactericidal in the higher concentrations tested; however, the difference between lethal and inhibitory levels is greater than with penicillin and streptomycin. Erythromycin is also a less efficient bactericidal agent than penicillin and streptomycin; more than twenty-five times the minimum inhibitory level is needed before the strains of enterococci are efficiently killed.

Table III shows the average blood levels which have been reported by various authors during the customary courses of antibiotic treatment; in

TABLE I. THE MINIMUM INHIBITORY CONCENTRATION OF 7 ANTIBIOTICS FOR 18 STRAINS OF ENTEROCOCCI

PENICILLIN		STREPTOMYCIN		ERYTHROMYCIN		BACITRACIN		CHLORTETRA-CYCLINE		OXYTETRA-CYCLINE		CHLORAMPHENI-COL	
NO. OF STRAINS	M.I.C. MCG./ML.	NO. OF STRAINS	M.I.C. MCG./ML.	NO. OF STRAINS	M.I.C. MCG./ML.	NO. OF STRAINS	M.I.C. MCG./ML.	NO. OF STRAINS	M.I.C. MCG./ML.	NO. OF STRAINS	M.I.C. MCG./ML.	NO. OF STRAINS	M.I.C. MCG./ML.
0	0.75	0	3.0	6	0.05	0	0.25	1	0.05	0	0.15	1	7.5
15	1.5	4	6.0	1	0.4	1	0.5	4	0.1	1	0.3	0	15.0
3	3.0	11	12.5	9	0.8	0	1.0	5	0.2	5	0.6	15	30.0
		3	25.0	2	1.6	8	2.0	8	0.4	11	1.2	2	60.0
						9	4.0			1	2.4		

exceptional cases it is possible to obtain higher concentrations and some of these maximum levels have been tabulated.[3, 10, 20-24] The important question is whether these levels are bactericidal for enterococci causing endocarditis. A comparison of the three tables shows that both bacteriostatic and bactericidal concentrations can be achieved in the blood with penicillin and streptomycin. Bacitracin and erythromycin are mainly inhibitory, bactericidal effects being exerted on only a few of the strains; while chlortetracycline and oxytetracycline are entirely inhibitory. Chloramphenicol would be rejected as a form of treatment even when judged by its inhibitory effect.

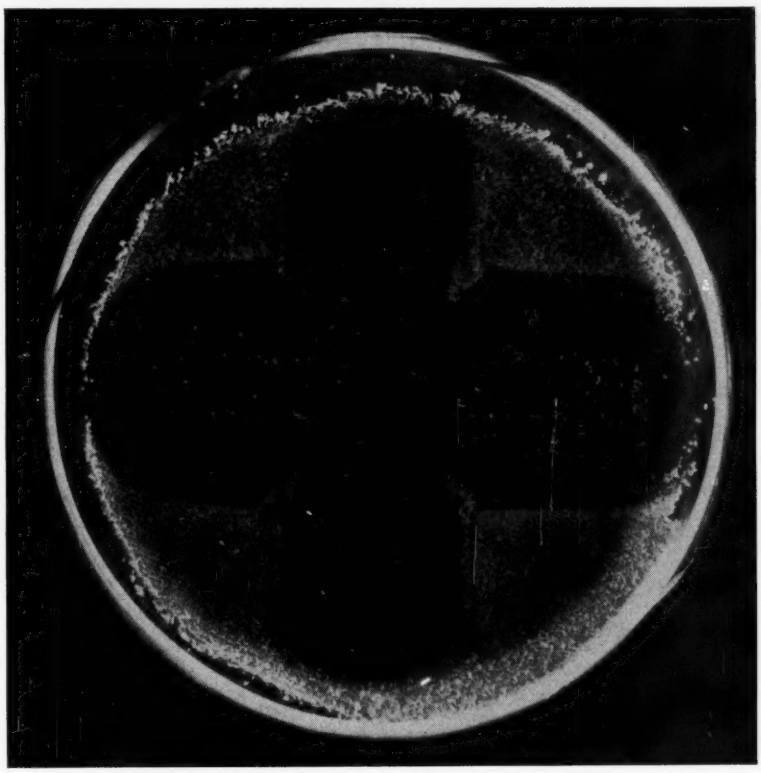

Fig. 4.—Replica plate illustrating the bactericidal effect of penicillin (horizontal) and the fine growth in the zone of chlortetracycline (vertical). Where both drugs act together the bactericidal effect of penicillin is annulled as shown by the presence of growth in the area.

The customary bactericidal sensitivity tests are carried out by exposing the organisms to several antibiotics in the hope of finding one which inhibits the growth in vitro, in concentrations that can be achieved in the body. It so happens that the bacteriostatic and bactericidal levels are close for penicillin and streptomycin but greatly different for the other drugs (Tables I and II). This explains why the routine test based on inhibitory effect proved useful for the assessment of penicillin and streptomycin, but misleading, in this disease, for chlortetracycline and oxytetracycline.

TABLE II. NUMBER OF STRAINS OF ENTEROCOCCI KILLED AT VARIOUS CONCENTRATIONS OF 7 ANTIBIOTICS

PENICILLIN		STREPTOMYCIN		ERYTHROMYCIN		BACITRACIN		CHLORTETRA-CYCLINE		OXYTETRA-CYCLINE		CHLORAMPHENI-COL	
NO. OF STRAINS	MCG./ML.	NO. OF STRAINS	MCG./ML.	NO. OF STRAINS	MCG./ML.	NO. OF STRAINS	MCG./ML.	NO. OF STRAINS	MCG./ML.	NO. OF STRAINS	MCG./ML.	NO. OF STRAINS	MCG./ML.
3	2	11	12	0	0.8	2	3	0	2.5	0	2.5	0	40
2	5	7	25	3	2	3	15	0	12.5	0	12.5	0	200
13	10			2	5	12	75	0	25	0	25	0	1000
				1	10			0	50	0	50		
				12	25								

TABLE III. AVERAGE AND MAXIMUM BLOOD LEVELS REPORTED DURING TREATMENT WITH THE 7 ANTIBIOTICS (MAXIMUM LEVELS IN ITALICS)

ANTIBIOTIC	DOSAGE	BLOOD LEVEL
Penicillin	1-4 mega units/day	1-10 units/ml.
	Over 20 mega units/day	*–150 units/ml.*
Streptomycin	1-2 Gm./daily	25-20 mcg./ml.
	1 Gm./ 6 hourly	*50-100 mcg./ml.*
Erythromycin	0.3 Gm./6 hourly	1-10 mcg./ml.
	0.5 Gm./6 hourly	*5-20 mcg./ml.*
Bacitracin	30,000 units/6 hourly	1-3 mcg./ml.
Chlortetracycline	0.5-0.75 Gm./6 hourly	1-10 mcg./ml.
	1.0 Gm./6 hourly	*10-20 mcg./ml.*
Oxytetracycline	0.5-0.75 Gm./6 hourly	1-10 mcg./ml.
	1.0 Gm./6 hourly	*10-20 mcg./ml.*
Chloramphenicol	0.5 Gm./6 hourly	5-20 mcg./ml.
	0.75-1.0 Gm./6 hourly	*25-50 mcg./ml.*

TABLE IV. INTERACTION OF BACTERICIDAL ACTION BETWEEN 7 ANTIBIOTICS ARRANGED IN PAIRS, ACTING ON 18 STRAINS OF ENTEROCOCCI

COMBINATION OF ANTIBIOTIC	SYNERGISM	ANTAGONISM	NO EFFECT
Penicillin + streptomycin	15	0	3
Penicillin + erythromycin	0	5	13
Penicillin + bacitracin	8	2	8
Penicillin + chlortetracycline	1	6	11
Penicillin + oxytetracycline	4	6	8
Penicillin + chloramphenicol	0	16	2
Streptomycin + erythromycin	7	0	11
Streptomycin + bacitracin	18	0	0
Streptomycin + chlortetracycline	8	0	10
Streptomycin + oxytetracycline	8	0	10
Streptomycin + chloramphenicol	17	0	1
Erythromycin + bacitracin	0	0	18
Erythromycin + chlortetracycline	2	1	15
Erythromycin + oxytetracycline	2	1	15
Erythromycin + chloramphenicol	1	1	16
Bacitracin + chlortetracycline	4	2	12
Bacitracin + oxytetracycline	12	0	6
Bacitracin + chloramphenicol	2	11	5
Chlortetracycline + oxytetracycline	0	0	18
Chlortetracycline + chloramphenicol	3	0	15
Oxytetracycline + chloramphenicol	8	0	10

The findings obtained for the 21 possible combinations of the drugs in pairs are shown in Table IV. Nine of these pairs show either synergism or no interaction; synergism occurs most frequently with penicillin + streptomycin, streptomycin + bacitracin, and streptomycin + chloramphenicol. Eight of the remaining pairs of antibiotics are either synergistic or antagonistic, according to the strain of enterococcus; antagonism is most consistently, though not invariably, demonstrated by the combination of penicillin + chloramphenicol. Erythromycin + bacitracin and chlortetracycline + oxytetracycline are exceptional in that neither synergism nor antagonism can be demonstrated, in the case of the latter pair, a result to be expected from their very close chemical similarity.

DISCUSSION

Subacute bacterial endocarditis presents special problems to both clinician and bacteriologist. It has become evident that in this disease the organism must be killed by the antibiotic and not just inhibited. Moreover, it is highly

probable that blood concentrations greater than the "minimum bactericidal level" are needed to enable the drug to diffuse into the lesions and maintain a local bactericidal activity. For this reason the choice of antibiotic is much more limited than in the treatment of most other infections, and the customary laboratory test for antibiotic sensitivity is inadequate and may in fact even be misleading. Our findings confirm that for penicillin and streptomycin the inhibitory concentrations are very close to those which are bactericidal, but for the newer antibiotics, the bactericidal and bacteriostatic levels are often very far apart, and that, therefore, a choice of drug based on an inhibitory test is liable to lead to clinical failure. Penicillin, indeed, owing to its virtual lack of toxicity for man, continues to occupy a special position in the treatment of this disease. This is emphasized by the fact that apparently "penicillin resistant" cases of enterococcal endocarditis may yet be cured with very high doses of the drug after bacteriostatic drugs have failed.[25] Oral erythromycin alone, even in high doses, has failed to cure the disease,[26] and this supports our findings that, for the majority of the eighteen strains of enterococci in this investigation, the bactericidal levels are beyond those obtainable in the blood.

The method described detected 99 per cent lethal effect, which is conventionally regarded as only moderate lethal activity. However, the results appear to correlate with clinical experience. This may be because the lethal activity of a drug depends on the time during which it acts, as well as its concentration. In the test, an arbitrary period of 24 hours is investigated, but during therapy, organisms are exposed continuously for several weeks to varying concentrations of the antibiotics. The ultimate bactericidal action is due to the accumulative effect of the lethal activity demonstrated in the test.

The results of interaction of antibiotics obtained with the method described are supported to some extent by clinical findings. The synergism of penicillin and streptomycin is well recognized and has had striking success.[9, 11, 27] The effective use of erythromycin and streptomycin together has also been reported, although in this case erythromycin was given in massive doses, part of the time by the intravenous route.[28]

The clinical significance of antagonism is, however, not established. Jawetz and Gunnison[29] have put forward the generalization, based on in vitro experiments, that combinations of bactericidal drugs with bacteriostatic drugs are antagonistic. Our tests tend to confirm this for penicillin but not for streptomycin. The reason for this discrepancy is partly due to the arbitrarily chosen incubation time of 24 hours; it has been shown that after 8 hours there is antagonism between streptomycin and a bacteriostatic drug, which on further incubation disappears and is replaced by synergism.[19, 30] The longer incubation time might well reflect more closely the course of events during the protracted treatment of subacute endocarditis; there are many reports of clinical cure with use of combinations of antibiotics which might be expected to be antagonistic.[31, 32] Apart from a single series of cases of pneumococcal meningitis,[33] there has been no reported clinical evidence of antagonism.

The usual method of testing the inhibitory sensitivity of organisms isolated from cases of bacterial endocarditis can no longer be accepted as of value. It is

essential to determine the bactericidal effect of the drug, and the method described has proved satisfactory. When two drugs are used together, the method will give an indication as to the effect of these drugs on the organism in vitro, although the clinical significance of these findings has yet to be finally assessed.

SUMMARY

With use of a method suitable for routine use, the bactericidal effect of seven antibiotics was tested in vitro against 18 strains of enterococci isolated from subacute endocarditis.

In the case of penicillin and streptomycin the bactericidal levels closely correspond with the bacteriostatic levels. Penicillin and streptomycin at concentrations which can be obtained clinically are predominantly bactericidal, while chlortetracycline, oxytetracycline, and chloramphenicol are essentially bacteriostatic. Routine laboratory tests fail to make this distinction. The method is also used to demonstrate synergism or antagonism between drugs. The significance of these findings for clinical practice is discussed.

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PATHOGENICITY AND ONCOLYTIC CAPACITY OF RI VIRUS STRAIN RI-67 IN MAN

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THE recently discovered RI viruses,[1-3] which are also called APC[4-7] or ARD agents,[8] have been shown[1-3, 9] to be responsible etiologically for the majority of cases in military populations of undifferentiated acute respiratory disease (ARD),[10, 11] nonstreptococcal exudative pharyngitis,[11] and of primary atypical pneumonia,[11] in which the test for cold agglutinins remains negative. These agents have also been found associated with illness in civilian populations referred to as pharyngo-conjunctival fever[4-7] and with certain cases of mesenteric lymphadenitis.[12] These viruses are heterogeneous antigenically,[1, 5, 6, 13] comprising a group of distinct serotypes[5, 6]; however, they elaborate a common group-specific "soluble" antigen which is readily separable from the virus[6, 14, 15] and which appears not to be of significance in immunity to RI disease.[2, 9]

The present study was carried out during the winter of 1954 with a dual purpose. The first was to determine the pathogenicity of the prototype virus RI-67 (type 4) for a group of adult persons with cancer given the agent by the respiratory route in an effort to reproduce the disease. The second was to find out whether the virus administered by various routes would exert a significant oncolytic effect on human cancer in cases of advanced neoplastic disease.

MATERIALS AND METHODS

Clinical.—The 14 patients employed in the study were volunteers of both sexes, between the ages of 21 and 71 years, who had advanced cancer which had failed to respond to all established methods of surgical, radiotherapeutic, and chemotherapeutic treatment. The cancer diagnoses are given in Table I. All the volunteers were aware of the experimental nature of the treatment and the expectation that acute respiratory infections might result.

The candidates for the study were tested first for presence of complement-fixing (CF) antibody against the RI-67 virus, and, with 2 exceptions, only those with no detectable antibody were accepted. Neutralization tests were not performed prior to virus inoculation, but tests made subsequently revealed that 4 additional patients had pre-existing neutralizing antibody. During the RI-67 study, all the patients were cared for by a special nursing staff under strict isolation.

The RI-67 virus employed in the study was grown in HeLa cell cultures[16] and was administered by the respiratory or intramuscular route, as summarized in Table I. Patients receiving the nebulized virus held the nebulizer in their mouths while 1.0 ml. of the inoculum was administered during inspiration only, over a 15- to 30-minute period. The spray was

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TABLE I. CLINICAL, VIRUS ISOLATION AND SEROLOGIC FINDINGS

PATIENT	AGE AND SEX	CANCER DIAGNOSIS	VIRUS INOC. ROUTE	CLINICAL RESPONSE		
				FEVER* (DEGREES F.)	OTHER	TUMOR
1	71 M	Sq. Ca. larynx	Nebul.	101.2	Tracheitis	None
2	58 M	Adenoca., stomach	Nebul.	None	None	None
3	63 F	Lympho-sarcoma	Nebul.	None	None	No data, died day 10
4	49 F	Adenoca., ovary	Nebul.		No data, died day 1	
5	29 M	Osteo-sarcoma	Nebul. and intraderm.	101.8	Anorexia, nausea and vomiting	None
6	53 M	Sq. Ca. bronchus	Nebul. and intraderm.	None	None	None
7	54 M	Sq. Ca. bronchus	Nebul. and intraderm.	100.6	None	None
8	43 F	Adenoca., breast	Nebul. and intraderm.	None	None	None
9	63 M	Sq. Ca. foot	Intranasal and intraderm.	100.8	None	None
10	21 M	Malignant thymoma	Intramusc.	101.0	None	Suggestion
11	55 F	Adenoca., breast	Intramusc. and intraderm.	101.6	None	Suggestion
12	43 F	Adenoca., uterus	Intramusc.		Not interpretable, died day 13	
13	53 M	Sq. Ca. lip	Intramusc. and intratumor	None	None	No data, died day 12
14	66 M	Sq. Ca. palate	Intramusc. and intratumor		Not interpretable, died day 7	

Sq. Ca. = Squamous cancer.
Adenoca. = Adenocarcinoma.
Nebul. = Nebulizer.
*Fever is the highest postinoculation temperature which was in excess of the preinoculation maximum.

administered directly into the trachea of one of the patients (Patient 1) who had a permanent tracheostomy. Following spraying, an additional 0.5 ml. was given by dropper directly into the nasopharynx. One individual was given 1.5 ml. of the virus by the nasal route alone. The nebulizer was powered by compressed oxygen for 3 patients and by compressed nitrogen for the remaining 5. Titration of the virus remaining in the nebulizer at the end of the spray period showed no detectable loss of infectivity when either gas was used. Five other patients were injected intramuscularly with 4.0 ml. of RI-67 culture, and certain of these also received 1.0 ml. into a superficial tumor.

Some patients (Table I) were also given 0.1 ml. of the virus and a like amount of normal HeLa control material intradermally on the forearm. This was done to test for allergic sensitivity, for local inflammatory reaction due to virus propagation, and for possible oncogenesis resulting from inoculation of HeLa cell material.

Criteria for clinical evaluation of virus infection included periodic physical examination of the nose, throat, and chest, recording of temperature, pulse, and respiration 4 times daily, and periodic blood counts, erythrocyte sedimentation rates, and throat cultures. For the virus isolation work, throat washings in veal infusion broth and heparinized samples of blood were collected just before administration of virus and daily for 14 days thereafter. Biopsy specimens of skin or tumor tissue were taken as indicated by clinical developments and the autopsy materials were collected 2 to 12 hours following death. All the specimens were frozen immediately in dry ice and sent to the Army Medical Service

IN 14 CANCER PATIENTS INOCULATED WITH RI-67 VIRUS

VIRUS ISOL. TESTS—DAYS AFTER INOCULATION					ANTIBODY TITERS‡			
THROAT WASHINGS	BLOOD	SKIN	RESPIRATORY TISSUE†	CANCER	TECH.	PRE-VIRUS	DAY (5-10)	DAY (11-45)
Neg. 1, 4, 8, 12					C. F.	0	0	0
					Neut.	0		32
Neg. 7					C. F.	0	0	20
					Neut.	0	0	2
Neg. 1			Neg. 10		C. F.	0	0	No data,
					Neut.	0	0	died day 10
			Neg. 1		C. F.	2	No data,	
					Neut.	0	died day 1	
Neg. 2, 7, 12		Neg. 6			C. F.	0	0	160
					Neut.	0	2	128
Neg. 1					C. F.	0	0	5
					Neut.	8		128
Neg. 4, 8, 11				Neg. 6	C. F.	0	0	40
					Neut.	8	32	512
Neg. 7		Neg. 4			C. F.	0	40	160
					Neut.	0	128	512
Neg. 1		Neg. 8			C. F.	0	0	10
					Neut.	2	8	32
Neg. 3	Neg. 3, 5, 7				C. F.	0	0	80
					Neut.	0		512
Neg. 4	Neg. 1, 2, 4, 6, 8	Neg. 9		Neg. 7	C. F.	0	0	40
					Neut.	0		512
			Neg. 13	Neg. 13	C. F.	2	5	20
					Neut.	2	32	128
				Pos. 5	C. F.	0	0	No data,
				Neg. 10	Neut.	0	128	died day 12
				Neg. 7	C. F.	0	No data,	
					Neut.	2	died day 7	

†Respiratory tissue included specimens of trachea, bronchi, and lung parenchyma obtained at autopsy.

‡Antibody titers designated as "0" were <1:2 in all neutralization tests and preinoculation C.F. tests and were <1:5 in postinoculation C.F. tests.

Graduate School where the laboratory studies were conducted. Blood samples for serologic investigations were taken prior to virus inoculation and at weekly intervals thereafter. The sera, removed from the clots, were stored frozen at –20° C. until tested.

The evaluation of antineoplastic effects was based on comparative observations made before and at periodic intervals after virus inoculation. The criteria included wherever possible (a) size, number, and character of tumor masses determined by physical examination and by photographic record, (b) alteration of function of organs damaged by the neoplastic disease as judged by clinical laboratory tests, and (c) comparative pathologic studies of biopsies or Papanicolaou smears.

Laboratory.—

Virus inoculum: The strain of RI-67 virus employed in the present work was isolated originally in cultures of human tracheal epithelium from the throat washings of a patient with primary atypical pneumonia, and after 4 passages in tracheal tissue, the virus was adapted to growth in HeLa cell cultures.[1] Virus from the eleventh passage in HeLa cells was used to prepare inoculum for the patients. For this purpose infected HeLa cultures were treated in a Waring blendor for 2½ minutes to disrupt the cells, after which the suspension was clarified by spinning for 10 minutes at 3,000 r.p.m. in the horizontal head of a No. 1 International Electric Clinical centrifuge. The supernatant fluid was centrifuged again and the final superfluent, which was the inoculum, was stored frozen at –70° C. in aliquots in sealed glass ampules. Two different lots of seed virus were employed as inocula in the experiments, each of which had an infectivity titer of 10^{-2} when tested in the usual manner.[1] The

fluid in which the tissue cultures* were maintained during viral growth and in which the cells were homogenized consisted of 10 parts pooled chicken serum, 5 parts 50 per cent chick embryo extract ultrafiltrate, 85 parts Hanks-Simms' solution, and 50 units and 50 μg of penicillin and streptomycin, respectively. Aerobic and anaerobic cultures of the seed virus on blood agar and in Brewer's thioglycollate medium failed to show the presence of bacterial contaminants, and tests to detect extraneous virus by inoculation of white mice, guinea pigs, and embryonated eggs gave negative results.

Virus recovery: The RI-67 virus isolation attempts were carried out in HeLa cultures with the employment of the same general methods described earlier.[1] Autopsy or biopsy specimens from the patients which had been stored frozen at −70° C. were triturated in a mortar with sufficient sterile veal infusion broth to give a 10 per cent suspension and were clarified by spinning at 2,000 r.p.m. in the horizontal centrifuge. One-tenth milliliter amounts of the superfluent obtained from the homogenized tissues or of the body fluids or of throat washings from the patients were inoculated into groups of 3 HeLa culture tubes. If no cytopathogenic effect was observed within 3 days' incubation at 36° C., the tissues and fluids were harvested, treated for 2 minutes in a Mickle Tissue Disintegrator,† centrifuged lightly to remove debris, and passed to new tubes. Cultures failing to show typical degeneration in 2 passages were discarded. Recovered virus was identified in serum neutralization tests with known positive antiserum.

Serology: Serum neutralization and complement-fixation (C.F.) tests with the RI-67 virus and the patients' sera were performed according to methods already described.[1] Serum titers were expressed as the greatest initial dilution of patients' sera which caused complete or nearly complete suppression of viral growth (neutralization) or of fixation of complement (C.F.).

RESULTS

Clinical Evidences of Virus Infection.—Clinical evidence of respiratory infection occurred in only one of the 14 patients (No. 1 in Table I and Fig. 1) following administration of RI-67 virus. This individual developed a mucous tracheitis, with fever of 100.8° F., on the seventh day after receiving nebulized virus directly into the trachea through a permanent tracheostomy. There was no pharyngitis and no physical or roentgenologic evidence of pneumonitis during the period of observation. The temperature returned to the usual level by the tenth day and the tracheal mucosa appeared normal on the fourteenth day. Virus isolation attempts gave negative results with tracheal secretions collected from Patient 1 on the first, fourth, eighth, and twelfth days. Bacterial cultures of tracheal secretions collected two days prior to virus inoculations and 11 and 17 days thereafter all yielded pneumococci in a mixed flora. The significance of these bacteria in the tracheitis could not be assessed, since the organisms were present before and after, as well as during, the tracheitis.

No other patient developed symptoms, physical signs, or roentgenologic evidence of respiratory tract infection. However, Patient 5 (Table I and Fig. 2), who received virus by nebulizer, developed low-grade fever, malaise, anorexia, nausea, and vomiting, beginning on the first day and persisting for about one week. Moreover, Patients 10 and 11 (Table I and Fig. 3), who were given the virus intramuscularly, developed low-grade fevers, with no other clinical evidences of viral infection. No obvious extraneous cause for

*The HeLa cell cultures and tissue culture fluids employed in these investigations were obtained from Microbiological Associates, Inc., Bethesda, Md.

†H. Mickle Company, Hampton, Middlesex, England.

these signs and symptoms was apparent and it was assumed that they were attributable to RI-67 virus infection. In all other patients there were neither signs nor symptoms of respiratory disease, nor was there systemic illness which could not be readily explained by the neoplastic disease.

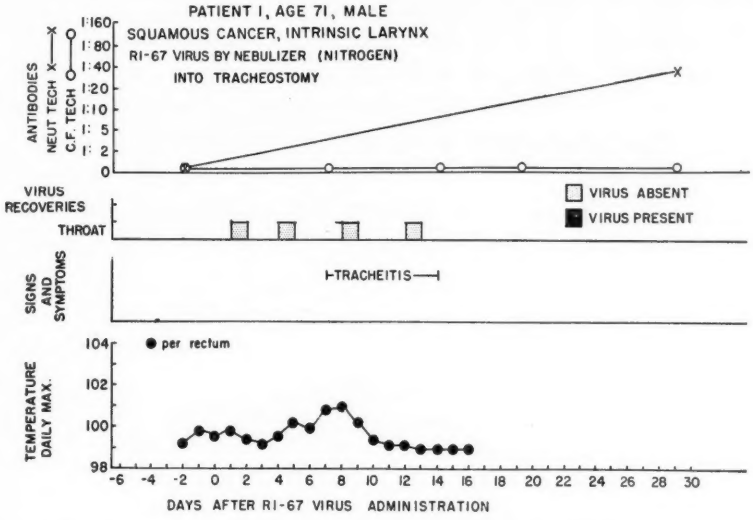

Fig. 1.—Occurrence of fever, tracheitis, and neutralizing antibody in Patient 1 following intratracheal inoculation of RI-67 virus.

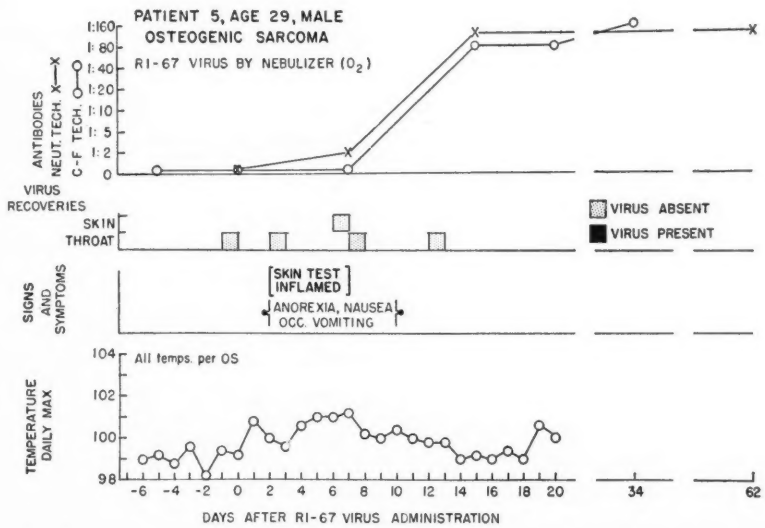

Fig. 2.—Occurrence of fever, gastrointestinal symptoms, and antibody formation following administration of virus by nebulizer and intradermally to Patient 5.

Each of the six persons who was inoculated intradermally with the RI-67 virus developed erythema 1 to 2 cm. in diameter at the injection site. This appeared in 48 to 72 hours, reached maximum intensity on the following day and disappeared during the next few days. Punch biopsies from the

inflamed skin of four patients showed diffuse infiltration of the dermis with inflammatory cells, chiefly mononuclear. The epidermis was not involved and inclusion bodies were not seen. In contrast to these findings, the site of inoculation of the noninfected HeLa cell control material showed neither macroscopic nor microscopic evidence of inflammation.

None of the biopsy tissues taken from the virus or control inoculation sites showed neoplastic change. Moreover, there was no evidence of tumor formation on inspection or palpation in any of the intradermal or intramuscular injection areas during the period of observation which lasted up to three months in certain cases.

Virus Isolation Attempts.—Forty-four specimens from the 14 patients were tested for the presence of RI-67 virus. The types of specimens and time of collection are indicated in Table I and Figs. 1 to 4. The virus was recovered from only 1 of the 44 specimens, i.e., the squamous cell carcinoma tissue of Patient 13 (Table I, Fig. 4), which had been inoculated five days prior to the biopsy. A biopsy taken simultaneously from a near-by tumor mass which had not been inoculated contained no demonstrable virus. It may be noted that this patient had received virus intramuscularly as well as directly into one of the tumor masses. Specimens of pleural fluid from Patient 3 (lymphosarcoma with pleural metastases), collected 2, 3, and 7 days after administration of virus by nebulizer, also failed to yield virus; this is not mentioned in Table I.

Serologic Studies.—Every patient who survived for at least 8 days after inoculation, developed or showed an increase in antibody against the RI agent detectable by the C.F. or neutralization technique or both. This included 2 patients (Nos. 1 and 2) who received virus by the respiratory route only. Time and magnitude of the antibody rises are indicated in Table I and Figs. 1 to 4. In 3 patients (Nos. 5, 10 and 13), contrary to the usual experience in the natural infection,[1, 9] the neutralizing antibody appeared earlier than the C.F. antibody. Patient 10 developed a neutralizing antibody titer of 1:512 on day 14, but the C.F. test did not become positive until day 45.

Oncolytic Effects.—There was no definitive evidence of oncolytic (antineoplastic) effect, but suggestive changes did occur in 2 persons given virus intramuscularly. One of these (Patient 11, Fig. 3) had primary inoperable cancer of the breast with local, axillary, cervical, bone, and brain metastases. The last named had been controlled by roentgenotherapy applied to the occiput one month previously; there had been no roentgenotherapy of other areas and no chemotherapy. Diffuse bony infiltration was implied by markedly negative calcium balance but was not demonstrable by roentgenograms. The changes which followed inoculation of virus and which suggested partial tumor regression were (a) decrease in peau d'orange appearance of the skin of the breast, (b) apparent shrinkage of palpable masses, and (c) significant decrease in calcium excretion, although a positive calcium balance was not attained.

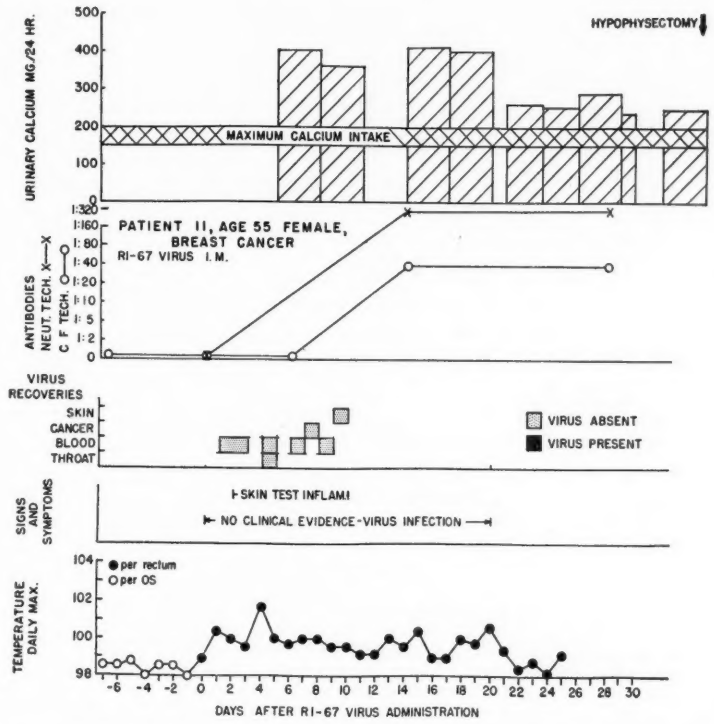

Fig. 3.—Development of fever, antibody response, and decrease in severity of negative nitrogen balance following intramuscular injection of virus in Patient 11.

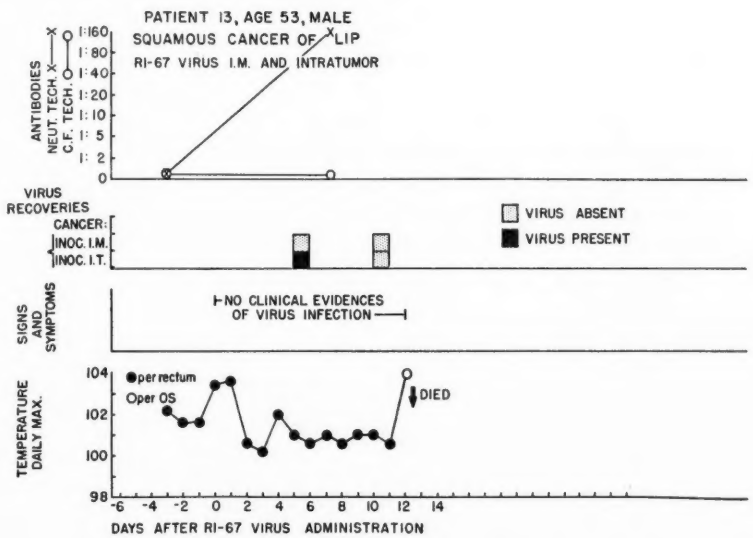

Fig. 4.—Identification of virus in inoculated tumor after five days, and absence of virus in uninoculated tumor following intramuscular and concomitant injection of cancer metastasis.

These changes began about 2 weeks after virus inoculation and continued through the remaining 3 weeks of observation, after which other experimental therapy was initiated.

Patient 10 had a malignant thymoma, with multiple subcutaneous metastases in the scalp and persistent severe headache of recent origin. Involvement of the spine had been treated with nitrogen mustard and local x-ray about 8 months previously, but the patient remained paraplegic. Throughout the second week after intramuscular virus inoculation, during a period of low-grade fever, there was diminution in size of palpable tumor masses and relief of the headache with resulting cessation of narcotic demand. During the third week the headache returned and renewed growth of tumors became obvious.

It is conceivable, but by no means certain, that the changes observed in these two patients were related to virus infection. Proof for virus propagation was not obtained, since the agent was not detectable either in the tumor biopsy obtained from Patient 11 on day 7 or in the blood of either of the two patients. However, the occurrence of fever and the appearance of RI antibody favored the assumption that the virus had propagated. There was no gross or microscopic evidence of oncolysis in the carcinoma of Patient 13 from which virus was isolated 5 days after intratumor inoculation.

DISCUSSION

The failure in the present study, as in previous investigations by Huebner and associates,*[5] to produce respiratory disease in the volunteers was unexpected in view of the evidence now at hand implicating these viruses in the etiology of acute respiratory illness.[1-9, 18] One possible explanation is that the virus might have lost its virulence for man while still retaining its antigenicity during the 15 passages in tissue culture. Another is that four of the volunteers had specific neutralizing antibody prior to inoculation and the others may have had small but undetectable amounts of these immune substances. Still another possibility is that the volunteers studied were older (aged 29 to 71) than newly recruited soldiers (aged 18 to 22), in whom the severe epidemic disease occurs naturally, and the former were not exposed to the stress of active military training. It should be noted also that recruit volunteers inoculated by Dingle and co-workers[18, 19] with throat washings from a patient with ARD developed only a mild disease and that even in epidemics of the natural disease among recruits in winter training, about one-half of the individuals who became infected fail to develop significant illness.[2, 9]

The objective evidences of slight and transient diminution in size and activity of cancer, which were observed in 2 of the 5 patients inoculated intramuscularly, are of interest. However, there is so much variation in the course of neoplastic diseases in individual patients that minor changes are notoriously inadequate and unreliable standards for evaluation of total cancer activity. Therefore, it must be concluded that the changes could not be considered as

*More recently, studies by Ward and associates[17] have shown that viruses of this family may cause fever, conjunctivitis, and respiratory tract involvement following conjunctival inoculation.

more than suggestive evidence of oncolysis. The demonstration of virus in a squamous cell carcinoma (Patient 13) after direct inoculation into the tumor suggested that the virus did proliferate in the cancer in vivo, but even in that patient there was no dissemination of virus to cancer tissues not directly inoculated and there was no significant destruction of the cancer cells. The production of a clinically worth-while destruction of metastatic cancer by a virus, or other systemically administered agents, would demand a generalized distribution of the agent to all possible sites of cancer involvement and selective destruction of the cancer cells. The absence of demonstrable virus in either blood or tumor tissue following intramuscular injection in the present study thus reduces the possibility that RI-67 virus has significant oncolytic potential. The administration of larger doses of virus intravenously to increase the chance of infection of tumor cells may merit further investigation. The possible oncolytic value of other serotypes of these respiratory viruses was not investigated.

Especially significant in the present study was the striking serologic response in the volunteers following injection of living virus. Thus, all of 9 patients receiving the agent by the dermal, muscular, or combined routes developed a high level of neutralizing antibody, 1:32 to 1:512 or greater, which is as great as that ordinarily achieved in the natural infection. Five of these persons had no detectable antibody in their preinoculation specimens. Both of the individuals who received virus by the respiratory tract alone, and lived for 26 days thereafter, developed antibody. This latter observation suggested that infection in the respiratory tract was established, although possible immunization by the primary inoculum dose cannot be excluded.

SUMMARY

Respiratory virus strain RI-67 in fifteenth passage in tissue culture was inoculated into fourteen volunteer patients, 29 to 71 years of age, with advanced incurable cancer. Among nine patients receiving virus by the respiratory route, two developed low-grade fever; one of these experienced a tracheitis and one had gastrointestinal symptoms. The inoculation procedure did not effect massive viral proliferation and the agent could not be recovered from the blood, secretions, or respiratory tissues. The virus was recovered once from a cancer which had been inoculated intratumor. There was no convincing evidence of cancer regression and no evidence of selective localization of virus in cancer tissue. Neutralizing and C.F. antibody appeared or increased in amount in all patients inoculated by respiratory or parenteral route who survived for at least nine days following injection.

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THE INFLUENCE OF DIET AND OF EXOGENOUS GLUCURONOLACTONE OR GLUCURONATE UPON THE URINARY AND SERUM GLUCURONIC ACID OF ADULT HUMAN SUBJECTS

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THE glucuronic acid that occurs naturally in body fluids and tissues is regarded as being present exclusively in the combined form as glucuronides or complex carbohydrates. Existing knowledge concerning the metabolism of exogenous glucuronic acid or its lactone is quite limited. Some data concerning the effect of glucuronolactone ingestion on the composition of blood and urine have been reported.[1, 2] Absorption appears to occur readily and only a small fraction of the ingested material is excreted in the urine. Tracer studies using C^{14}-labeled glucuronolactone indicate oxidation as evidenced by the excretion of radioactive CO_2 by the lungs.[3] There is less information available concerning the absorption, metabolism, and excretion of the salt form of this acid. C^{14} studies on animals injected with labeled glucuronate indicate less oxidation of this compound to CO_2 than of the lactone.[4]

The present study on human subjects was undertaken to compare the effect of ingestion or injection of glucuronolactone or glucuronate on the composition of blood serum and urine. Preliminary studies were made on the reliability of existing methods for estimation of glucuronic acid in blood plasma and urine and upon the stability of the compounds in serum and urine. It also seemed important to determine whether or not the protein content of the diet significantly influenced the excretion of glucuronides in the urine. In order that gastrointestinal absorption might be excluded as an unknown variable in the fate of these compounds, studies were made of their urinary excretion and serum concentration following the intravenous infusion of comparable amounts of glucuronic acid as its lactone or as sodium or calcium glucuronate.

EXPERIMENTAL METHODS AND RESULTS

The method of estimation of glucuronic acid as described by Maughan, Evelyn, and Browne[5] was modified in certain respects by Smith[16] and has been further modified in the present study to assure greater uniformity of the analytic procedure.

Various methods of deproteinizing plasma or serum were compared and lowest values were obtained when tungstate was used as the precipitating agent. Some increase in the apparent glucuronic acid content of the serum appears to occur after it has stood for 24 hours or longer at 4° C. It is therefore recommended that all filtrates be prepared on the same day that blood is drawn and that the determination be carried out not later than the second day.

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Normal Excretion of Glucuronic Acid.—The daily urinary excretion of glucuronic acid was determined on 9 adult males for 5 consecutive days. Chloroform was used as a preservative and each 24-hour specimen ended at 7 A.M. The daily excretion for a given subject was found to be quite constant (average ± 23 mg.) and the average value for the group was 449 mg. per 24 hours. The diurnal variation in glucuronic acid excretion was also determined on 5 subjects. These results indicated that some variation in the hourly excretion does occur. The lowest excretion rate was between 10 P.M. and 7 A.M. for the 4 male subjects, while the time of the highest rate of excretion differed.

Effect of the Protein Content of the Diet on Glucuronic Acid Excretion.—A comparison was made of the glucuronic acid excretion in 5 healthy male subjects maintained on low (0.7 Gm. per kilogram) and high (1.7 Gm. per kilogram) protein diets. These subjects were fed a diet constant in calorie and fat content, prepared in the metabolic kitchen, but otherwise continued their normal daily activities as employees of the hospital. Each diet was weighed carefully and aliquots were analyzed for their nitrogen content. All subjects were maintained on each diet one day prior to collection of specimens for analysis. Four 24-hour specimens were analyzed separately on each diet. The average excretion of glucuronic acid on the low protein diet was 0.439 Gm. and on the high protein diet 0.462 Gm. per 24 hours.

The general trend was toward a slightly higher glucuronic acid excretion (5 per cent increase) on a high protein intake, although the effect of approximately doubling the protein intake was relatively slight and might be regarded as negligible under ordinary circumstances.

The Effect of Ingestion of Glucuronolactone Upon Its Excretion in the Urine.—Four healthy male adults and two male patients were used for this study. The total daily dose was divided into 4 equal portions and taken before meals and at 10 P.M. The daily intake and urinary excretion of glucuronic acid are shown in Table I. The control excretion for each subject is based on an average of 3 separately analyzed 24-hour urine specimens, collected just prior to the period of glucuronolactone ingestion. The excretion of exogenous glucuronic acid is estimated by subtracting the control values from the total excretion during glucuronolactone ingestion. The healthy subjects excreted a fairly constant fraction of the amount ingested (0.1 Gm. per kilogram), ranging from 11.6 to 14.4 per cent (average 12.7 per cent). The two patients excreted a smaller fraction of the exogenous lactone (Patient A had recurrent renal stone formations; Patient B had advanced osteoporosis).

In another instance the daily excretion was followed in a healthy male subject who took progressively larger doses of the glucuronolactone until the total daily dose was 1.0 Gm. per kilogram or 10 times that of the other subjects. The per cent of each dose that was excreted in the urine remained remarkably constant as the dose was progressively increased (Table II). No untoward effects or subjective complaints were associated with the ingestion of the large doses of glucuronolactone by this subject.

TABLE I. URINARY EXCRETION OF GLUCURONIC ACID FOLLOWING INGESTED GLUCURONOLACTONE

SEX	AGE	CONTROL AVERAGE	GM. INGESTED*	DAILY EXCRETION OF GA IN GM. 1	2	3	4	AVERAGE	PERCENTAGE EXOGENOUS GA EXCRETED
M	28	0.581	9.5	1.824	1.787	1.892		1.834	13.1
M	29	0.548	7.0	1.453	1.441	1.625	1.711	1.557	14.4
M	30	0.450	7.4	1.311	1.172	1.373	1.441	1.324	11.8
M	38	0.513	7.6	1.504	1.022	1.429	1.634	1.397	11.6
Average		0.523	7.8	1.523	1.355	1.579	1.595	1.528	12.7
M-A	42	0.445	8.0	1.530	1.159	1.128	1.139	1.239	9.9
M-B	55	0.513	7.5	1.072	1.102	0.992	1.015	1.045	7.1

*0.1 Gm. per kilogram of body weight.

TABLE II. URINARY EXCRETION OF GLUCURONIC ACID FOLLOWING THE INGESTION OF GLUCURONOLACTONE IN PROGRESSIVELY LARGER AMOUNTS

Gm. Ingested daily	0.0	12.0	20.0	32.0	44.0	67.0	0.0
Gm./kg. Body weight	0.0	0.18	0.30	0.48	0.66	1.00	0.0
Gm. Excreted daily	0.366	2.318	3.643	5.790	6.540	12.166	0.3
% Exog. excreted		16.2	16.3	16.9	14.0	17.6	

TABLE III. URINARY EXCRETION OF GLUCURONIC ACID FOLLOWING ORALLY-INGESTED SODIUM GLUCURONATE*

SEX	AGE	CONTROL AVERAGE	GM. INGESTED	DAILY EXCRETION OF GA IN GM. 1	2	3	AVERAGE	PERCENTAGE EXOGENOUS GA EXCRETED
M	34	0.309	10.8	0.328	0.288	0.296	0.304	0.0
M	36	0.399	7.5	0.388	0.366	0.377	0.377	0.0
M	63	0.330	10.0	0.402	0.301	0.360	0.354	0.28
Average		0.346	9.4	0.372	0.318	0.344	0.345	0.09
M-A	42	0.445	8.0	0.544	0.501	0.461	0.502	0.08
M-B	55	0.513	7.5	0.569	0.467	0.484	0.506	0.0

*0.1 Gm. per kilogram of body weight.

Effect of Sodium Glucuronate Ingestion Upon the Urinary Excretion of Glucuronic Acid.—Three healthy adult male subjects and 2 patients ingested 0.1 Gm. per kilogram of glucuronic acid as the sodium salt. Urine analyses before and during the experimental period demonstrate no difference in glucuronic acid excretion during the time of glucuronate ingestion (Table III). No diarrhea or subjective complaints developed in any of the subjects given this dosage of sodium glucuronate.

Urinary Excretion of Glucuronic Acid Following the Intravenous Injection of Glucuronolactone or Calcium Glucuronate.—Two male patients, both of whom had advanced osteoporosis, maintained in the metabolic unit, were infused daily with 0.1 Gm. per kilogram of glucuronolactone. The daily dose of glucuronolactone was dissolved in 500 ml. of isotonic saline and infused at a constant rate over a 2-hour period. In some experiments, the urine samples were pooled for the 24-hour period, while in other instances the individual samples collected at 2-hour intervals were analyzed and the rate of excretion determined.

The results following glucuronolactone infusion are shown in Table IV. These data indicate that slightly more than half of the infused lactone was excreted in the urine. The results shown in the lower part of Fig. 1 indicate that the rate of excretion of the infused lactone was very rapid and that the urinary excretion rate approached the control value by the end of 7 hours following completion of the infusion. When calcium glucuronate was infused,

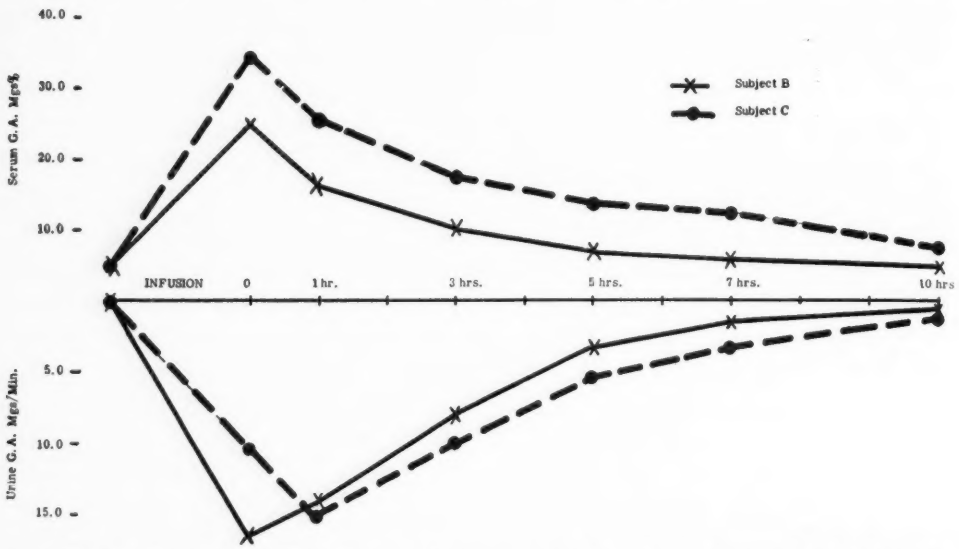

Fig. 1.—Upper half: The effect of glucuronolactone or calcium glucuronate infusion (0.1 Gm./Kg.) upon the serum concentration of glucuronic acid. Lower half: Corresponding studies on the rate of urinary excretion of glucuronic acid at the time of these infusions. Each subject gave identical curves for the two compounds so only 1 line is shown for both substances.

it was found that the decline in the serum concentration was essentially the same as for the glucuronolactone. The curves for the excretion rate of the two compounds were identical for a given subject but slightly different for the two subjects. The per cent recovery of glucuronic acid in the urine was consistently greater when glucuronate was infused than when the lactone was injected in similar experiments (see Tables IV and V).

TABLE IV. URINARY EXCRETION OF GLUCURONIC ACID FOLLOWING INTRAVENOUS INFUSION OF GLUCURONOLACTONE

SUBJECT		CONTROL	GM.	DAILY EXCRETION OF GA IN GM.						PERCENTAGE EXOGENOUS GA
SEX	AGE	AVERAGE	INFUSED	1	2	3	4	5	AVERAGE	EXCRETED
M-B	55	0.455	7.65	4.930	4.556	4.832	4.837	5.217	4.874	57.7
M-C	58	0.475	7.65	4.929	5.284	4.878	4.748		4.959	58.5

TABLE V. URINARY EXCRETION OF GLUCURONIC ACID FOLLOWING INTRAVENOUS INFUSION OF CALCIUM GLUCURONATE

SUB-JECT	CON-TROL AVER.	GM. IN-FUSED	1	2	3-6	7	8	9	10	11	AVER.	PERCENTAGE EXOGENOUS GA EXCRETED
B	0.513	6.86	6.308	5.687	6.299	6.044	5.795	5.768	6.098	5.991	5.998	87.4

All values expressed as glucuronolactone.

The Effect of Glucuronolactone or Glucuronate Ingestion or Injection Upon the Serum Concentration of Glucuronic Acid.—Five healthy, fasting male adult subjects were used for these observations. A control study consisted in drawing blood samples before and at specified time intervals after the ingestion of 400 ml. of tap water (Fig. 2). The serum values were found to be quite con-

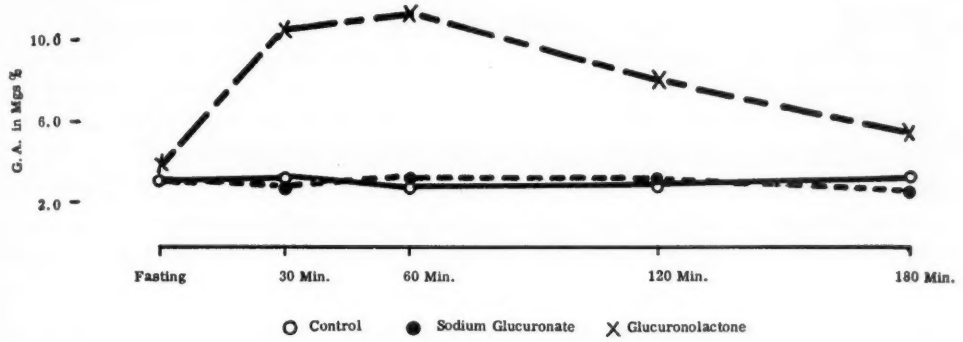

Fig. 2.—Serum glucuronic acid response to ingested glucuronolactone or glucuronate (0.1 Gm./Kg.).

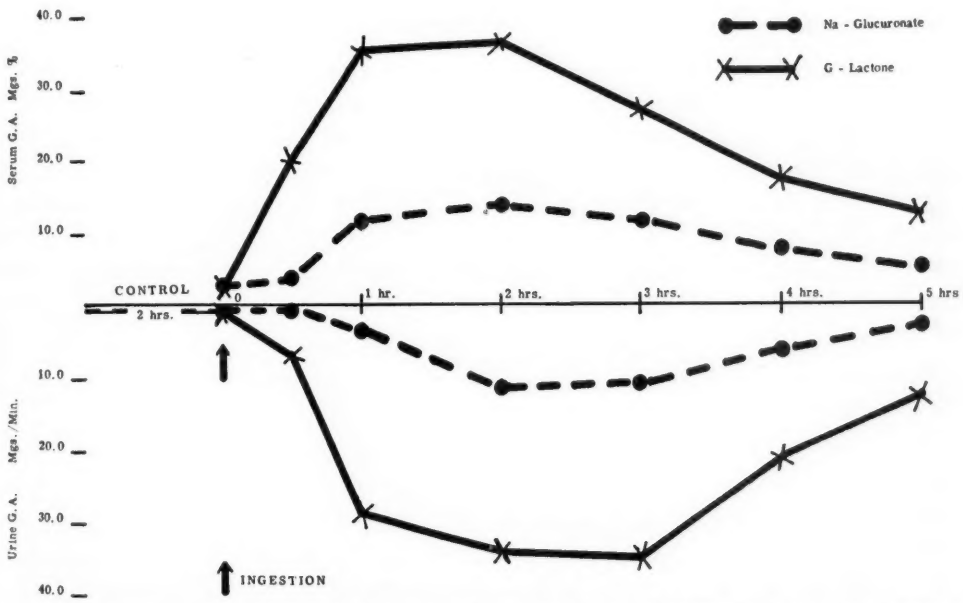

Fig. 3.—Serum concentration and urinary excretion rate of glucuronic acid by a fasting normal male subject following ingestion of 36.24 Gm. of glucuronolactone and 48.17 Gm. of sodium glucuronate (0.5 Gm. of glucuronolactone per kilogram of body weight).

stant. On a different day a similar study was made, except that the subjects ingested 0.1 Gm. per kilogram of body weight of glucuronic acid, either as the lactone or its sodium salt dissolved in 400 ml. of tap water.

The average values shown in Fig. 2 indicate a rapid rise in serum glucuronic acid following the ingestion of glucuronolactone, with a return to a range ap-

proximating the control values after 3 hours. The serum values did not change after sodium glucuronate ingestion and were identical with the control values for these subjects.

The blood concentration and urinary excretion following ingestion of a much larger amount of glucuronolactone or sodium glucuronate were studied in a healthy male subject. These data indicate (Fig. 3) that the glucuronate is absorbed but apparently at a much slower rate than is the lactone. Both substances were rapidly excreted in the urine in proportion to their plasma concentrations. Abdominal cramps and diarrhea were experienced 1 to 2 hours following ingestion of the large dose of sodium glucuronate but not after the lactone.

The rate of disappearance from the circulation of infused glucuronolactone or sodium glucuronate was studied in Subjects B and C. The rates of decline of the serum concentration of the two compounds infused at the same rate into a given subject were identical and are represented by a single line in Fig. 1. The rate of decline of the serum concentration in the two subjects differed somewhat and paralleled the differences observed in the rate of excretion in the urine of the two subjects.

DISCUSSION

Study of the method for the estimation of glucuronic acid in serum and urine has made apparent the need for greater specificity and stability of the chromogenic reagent, naphthoresorcinol. Substances other than glucuronic acid produce color with this reagent as applied to a serum or diluted urine. Correction factors have been recommended to correct for the color development due to glucose.[6] In our experience the effect of glucose on color development varies greatly with the glucose concentration and may reduce or enhance the amount of color obtained from a given amount of glucuronic acid.

The normal values obtained in this study for the daily excretion of glucuronic acid in the urine and for the serum concentration agree in general with those reported in the literature.[1, 6-10]

A high protein intake might be expected to contribute to the amount of phenol and similar compounds to be conjugated and excreted. The extent of intestinal putrefaction may be of more importance than the actual protein content of the diet. The slightly higher glucuronide excretion demonstrated to occur on a high protein intake agrees qualitatively with the report of others,[1] although quantitatively the effect of diet was so slight in the present study as to be negligible for all practical purposes. Ingestion of caffeine-containing beverages or drugs, such as acetyl salicylic acid, is much more apt to cause marked variations in glucuronide excretion than are variations in the composition of the diet.

There appears to be a significant diurnal variation in glucuronide excretion. The minimum excretion time for glucuronic acid coincides with the time

when the plasma and the urinary steroid values are lowest.[11-14] The nature of all the urinary glucuronides is not completely understood. Plasma and urine are ordinarily regarded as containing only conjugated glucuronic acid, except when exogenous glucuronic acid is administered. Unpublished observations with use of anion resins and paper chromatography confirm this impression for urine.

The prompt rise in the serum concentration of glucuronic acid following glucuronolactone ingestion is evidence for a rapid intestinal absorption of this compound. Similar observations regarding the serum response to ingested lactone have been reported by others.[1, 2] That the absorbed compound is rapidly metabolized is evidenced by excretion of radioactive carbon dioxide after C^{14}-labeled lactone is administered to animals[3] and by the relatively low excretion of ingested lactone in the urine. That the renal clearance rate for the lactone is high is evidenced by the fact that approximately 60 per cent of the injected material can be recovered in the urine. This value for human subjects in the present study agrees with reported values for the excretion of lactone injected into dogs.[15] Unpublished preliminary studies from this laboratory indicate that the renal clearance of glucuronolactone infused into dogs is essentially the same as the glomerular filtration rate.

The fact that a relatively constant fraction of ingested lactone is excreted in the urine, regardless of the dose, suggests that a balance existed between the rate of absorption and metabolism. This result does not agree with findings of a similar test reported by Hollmann,[15] who found that the per cent of the oral doses excreted decreased after the oral dose reached a certain value, presumably due to saturation of the absorption mechanism. That the glucuronolactone ingested is not excreted as the lactone but as the salt is suggested by the uptake of urinary glucuronic acid by an anion exchange resin in the acetate form, following glucuronolactone ingestion. This topic will be considered in greater detail in a subsequent publication.

The results following the ingestion of 0.1 Gm. per kilogram of sodium or calcium glucuronate gave no evidence of absorption as judged by the glucuronic acid content of serum or urine. Reported studies on the urine and expired carbon dioxide of animals injected with C^{14}-labeled glucuronate indicate that very little of the injected material was oxidized to carbon dioxide and that most of the activity could be accounted for in the urine.[4] That glucuronate is absorbed to some extent by man is evidenced by the blood and urine increase following ingestion of a single large dose by a fasting subject (Fig. 3).

The rapid excretion of injected glucuronate observed in the present study indicates a rapid clearance by the kidney. The fact that a higher proportion of injected glucuronate (81 per cent and 87 per cent) than of glucuronolactone (58 per cent) was recovered in the urine would suggest that either the salt has a higher clearance rate or that it was less rapidly metabolized than the lactone. Since both compounds are rapidly excreted, the extent to which they may be metabolized can hardly be judged from such experiments. The

in vivo rate of conversion of the lactone into the salt must also be a factor in its fate following ingestion. Whether or not enzymatic mechanisms catalyze this conversion in vivo remains to be determined. It seems evident from the differences in the in vivo behavior of glucuronolactone and glucuronate that the mammalian organism makes a clear distinction between the structure of glucuronolactone and that of the free acid or its salt.

SUMMARY

The rate of glucuronic acid excretion by healthy adult subjects was least during the night. The protein content of the diet has but slight effect on glucuronic acid excretion in the healthy adult human being. Ingestion of (0.1 Gm. per kilogram) glucuronolactone was followed by a prompt increase in the serum concentration. Less than 20 per cent of the ingested glucuronolactone was excreted in the urine, even when the intake was extremely high. Ingestion of 0.1 Gm. per kilogram of sodium glucuronate failed to alter the serum concentration or daily excretion of glucuronic acid in the urine of healthy adult subjects. However, a large oral intake (0.5 Gm. per kilogram) of the glucuronate significantly increased its content in blood and urine. Infusion of glucuronolactone or sodium or calcium glucuronate was followed by a rapid excretion of infused material in the urine. Sixty and eighty per cent of the infused lactone or salt, respectively, was recovered in the urine.

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SEVERE COAGULATION DEFECT WITHOUT HEMORRHAGIC SYMPTOMS CAUSED BY A DEFICIENCY OF THE FIFTH PLASMA THROMBOPLASTIN PRECURSOR

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RECENTLY Ratnoff[1, 2] presented three patients with virtually no hemorrhagic symptoms who had a disturbance of coagulation similar to the defect encountered in the hemophilia group. The clotting defect could be corrected with the plasma of all known subtypes of hemophilia: antihemophilic globulin (AHG) deficiency, plasma thromboplastin component (PTC) deficiency,[3] plasma thromboplastin antecedent (PTA) deficiency,[4] and deficiency of the fourth thromboplastin component of Spaet and associates.[5] Ratnoff, therefore, postulated the existence of a new disturbance of coagulation which he named Hageman's trait, after the first patient studied by him.

This is a report of two patients whose clinical and laboratory features were similar to the findings described in Hageman's trait: they had no hemorrhagic symptoms and the discovery of the clotting disturbance was incidental to a routine preoperative determination of bleeding time and clotting time.

METHODS

Collection and Processing of Blood and Plasma.—Silicone-coated syringes, needles, test tubes, and pipettes were used for all experiments, except for the determination of the venous clotting time and the prothrombin consumption test, which were performed in plain glass tubes. For the preparation of plasma, blood was mixed with $\frac{1}{10}$ volume of 0.1 M sodium oxalate or 3.8 per cent sodium citrate solution, depending on the test to be performed.

Bleeding Time.—Method of Ivy.[6]

Coagulation Time of Venous Blood.—One milliliter of blood was placed in each of two glass tubes (75 by 8 mm.) and transferred to a water bath of 37° C. Ten minutes after venipuncture, the first tube was tilted every minute until clotted. The second tube was started when the endpoint of the first one was reached. The endpoint of the second tube was recorded as clotting time of venous blood.

Prothrombin Time.—One-stage method of Quick.[7]

Recalcification Time.—Determination of the time interval between the addition of 0.1 ml. of 0.025 M $CaCl_2$ solution to 0.1 ml. of oxalated plasma and the appearance of a clot.

Fibrinogen Determination.—Method of Ratnoff.[8]

Platelet Count.—Method of Rees-Ecker.[9]

Capillary Fragility.—Rumpel-Leede Test.[10]

Clot Retraction.—Observation of clot incubated at 37° C. for 12 hours.

Prothrombin Concentration.—Modified two-stage method.[11]

Prothrombin Consumption.—Blood collected in a siliconized syringe was distributed into a series of 75 by 8 mm. dry glass tubes, calibrated to 1.0 ml. All tubes were placed in a

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water bath at 37° C. At regular intervals, 0.1 ml. of 3.8 per cent sodium citrate solution was added to successive tubes and the citrated serum was obtained by centrifugation. One-stage and two-stage determinations of prothrombin were performed on each sample of serum.

Thrombin Time.—Determination of the time interval between the addition of 0.1 ml. of a standardized solution of thrombin to 0.1 ml. of oxalated plasma and the appearance of a clot.

Normal $BaSO_4$-Treated Plasma.—This was prepared by adding 100 mg. of $BaSO_4$ (Merck and Company, Inc.) to each milliliter of normal oxalated plasma. This mixture was gently shaken for five minutes and $BaSO_4$ centrifuged out at 2,000 r.p.m. for ten minutes.

Normal Serum.—This was obtained from normal blood collected under sterile conditions and allowed to clot at 37° C. for forty-eight hours. Difco rabbit-brain thromboplastin and Armour's bovine fibrinogen were used throughout.

CASE REPORT

CASE 1.—R. L. (Minneapolis V. A. H. No. U-42527), a 32-year-old man, a bookkeeper, was first seen in 1948 because of low back pain of many years' duration. During hospitalization the patient requested the removal of a sebaceous cyst from his back. With no history of excessive bleeding the excision was accomplished uneventfully. The cyst measured 18 mm. in diameter. At the end of the procedure the surgeon became aware of the routine preoperative clotting time which was 72 minutes. However, there had been no increased bleeding during the procedure, and none occurred postoperatively. The patient was readmitted to the hospital in 1950 for extraction of three molar teeth, again without hemorrhagic complications. The past history revealed that the patient suffered a gunshot wound, in World War II, in the right shoulder which required hospitalization for approximately three weeks. Neither the wound nor the surgical procedure necessary for its care were complicated by any unusual hemorrhage. He underwent tonsillectomy as an adult without excessive bleeding. There was no family history of hemorrhagic diathesis. The physical examination was essentially negative.

Laboratory Tests.—The tests of hemostasis are presented in Table I and will be discussed later. Urinalysis was negative, hemoglobin 15.9 Gm. per cent, and leucocytes 6,500 per cu. mm. with a normal differential count. The bone marrow showed a normal morphologic picture. Erythrocyte sedimentation rate was 4 mm. per hour. Total serum proteins were 7.2 Gm. per cent, albumin 5.2 Gm. per cent, and globulin 2.0 Gm. per cent. Serum bilirubin prompt direct was 0.1 mg. per cent, and total 0.5 mg. per cent. Cephalin cholesterol flocculation showed a trace. Thymol turbidity was 3 units, bromsulfalein retention 3.5 per cent after 45 minutes, and serum cholesterol 224 mg. per cent.

CASE 2.—C. A. (Minneapolis V. A. H. No. U-94679), a 35-year-old man, a laborer, was admitted to the hospital on May 5, 1954, because of delirium tremens. The patient recovered rapidly, and on May 18, plans were made for a needle biopsy of the liver. However, the patient was found to have a clotting time of 50 minutes and the procedure was cancelled. The past history revealed that he had a tonsillectomy and adenoidectomy at the age of 9 years, an appendectomy at age 11, and several teeth extracted in adult life without any hemorrhagic complications. He never bled excessively from any cuts he contracted at work. The family history was negative for hemorrhagic diathesis. The patient had no abnormal physical findings after he recovered from the delirium tremens and there was no laboratory evidence of parenchymal liver dysfunction at the time of discharge from the hospital on June 11, 1954. The patient was observed for approximately one year, during which period there was essentially no change in the abnormal tests of hemostasis detailed in Table I.

Laboratory Tests.—Urinalysis was negative, hemoglobin 15.4 Gm. per cent, leukocytes 4,550 with a normal differential count, and erythrocyte sedimentation rate 7 mm. per hour. Total serum proteins were 6.5 Gm. per cent, albumin 4.0 Gm. per cent, and globulin 2.5 Gm. per cent, cephalin cholesterol flocculation was negative, thymol turbidity was 2 units, bromsulfalein retention 5 per cent after 45 minutes, and serum cholesterol 227 mg. per cent.

STUDY OF THE CLOTTING DEFECT

The two patients had identical abnormal tests: both had a prolonged clotting time, a prolonged recalcification time, and a markedly decreased prothrombin consumption. The other tests were all normal (Table I). This indicates that the clotting defect was located in the phase of thromboplastin formation. The analogy to the abnormalities encountered in the hemophilia group made it mandatory to determine if the two patients had a deficiency of any of the known plasma precursors of thromboplastin or if one was dealing with a defect of a new clotting factor. The possibility of a clotting inhibitor had also to be considered. Various types of mixing experiments were carried out in an attempt to approach this problem. In the first group of tests (Table II and Fig. 1) normal plasma and plasmas from patients with AHG deficiency, PTC deficiency, and PTA deficiency all corrected the clotting time and the consumption of prothrombin of patient R. L. These findings excluded an anticoagulant in the blood of R. L. and indicated that the abnormality was caused by a defect of a clotting factor different from AHG, PTC, and PTA. The plasma of patient C. A. tested on the blood of patient R. L. was ineffective, thus proving that both patients had the same defect. This was confirmed by adding the plasmas of the various types of hemophilia to the blood of Patient C. A.: all of them corrected the clotting time, and the consumption of prothrombin became normal. In this first group of tests the results obtained with the one-stage (Table II) and two-stage method (Fig. 1) for prothrombin determination were comparable. A reverse type of experiment is illustrated in Table III and

TABLE I. TESTS OF HEMOSTASIS

		PATIENT R. L.	PATIENT C. A.	NORMAL
Bleeding time	(min.)	4	4	Up to 6
Clotting time	(min.)	65	60	Up to 20
Plasma prothrombin time	(sec.)	13.3	13.4	12.0-14.0
Serum prothrombin time—2 hours after venipuncture	(sec.)	14.0	13.4	Above 23.0
Recalcification time	(sec.)	510	240	Up to 180
Platelets	(per cu. mm.)	192,000	252,000	150,000-250,000
Clot retraction		Normal	Normal	
Rumpel-Leede test		Negative	Negative	
Prothrombin concentration	(units/ml.)	360	345	260-360
Fibrinogen	(mg.%)	332	290	250-350
Thrombin time	(sec.)	23.8	25.0	23.0-27.0

TABLE II. EFFECT OF PLASMAS FROM INDIVIDUALS WITH AHG, PTC, AND PTA DEFICIENCY ON CLOTTING TIME AND PROTHROMBIN CONSUMPTION (ONE-STAGE METHOD) OF PATIENTS R. L. AND C. A.

PREPARATION MIXED WITH 1.0 ML. OF BLOOD OF PATIENT	PATIENT R. L.			PATIENT C. A.		
	CLOTTING TIME IN MINUTES	SERUM PROTHROMBIN TIME IN SEC.		CLOTTING TIME IN MINUTES	SERUM PROTHROMBIN TIME IN SEC.	
		1 HR.	2 HR.		1 HR.	2 HR.
0.1 ml. 0.9% NaCl sol.	41	13.4	13.2	60	11.3	13.4
0.1 ml. normal plasma	16	23.4	23.4	16	29.3	30.1
0.1 ml. AHG deficient plasma	12	25.0	40.0	16	41.0	46.8
0.1 ml. PTC deficient plasma	13	21.4	23.6	17	29.8	35.4
0.1 ml. PTA deficient plasma	13	21.6	23.7	17	25.2	30.6
0.1 ml. plasma of patient C. A.	39	13.6	13.7			

in Figs. 2, 3, and 4. In this second group of tests the plasmas of Patients R. L. and C. A. were tested on the blood of individuals with AHG deficiency, PTC

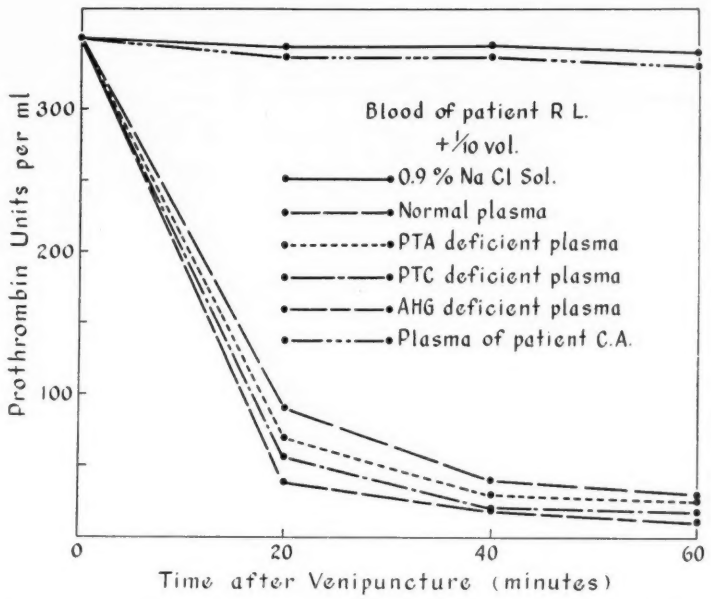

Fig. 1.—Effect of various plasmas on the consumption of prothrombin of Patient R. L. (two-stage method).

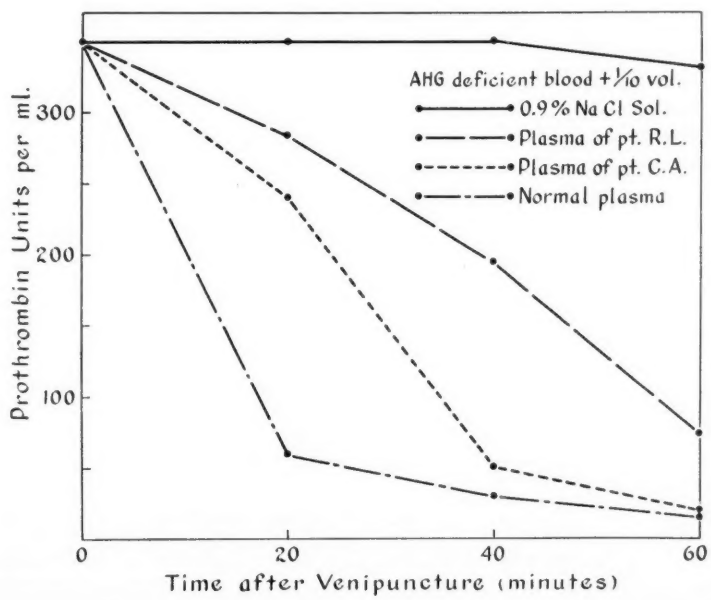

Fig. 2.—Effect of the plasmas of Patients R. L. and C. A. versus normal plasma on the consumption of prothrombin of AHG deficient blood (two-stage method).

deficiency, and PTA deficiency. The prolonged clotting times of the various subtypes of hemophilia were brought within normal limits by the plasma of

both patients. However, the effect of the same plasmas on the very poor consumption of prothrombin of the subgroups of hemophilia was inferior to the effect of normal plasma. This was especially true for the prothrombin consump-

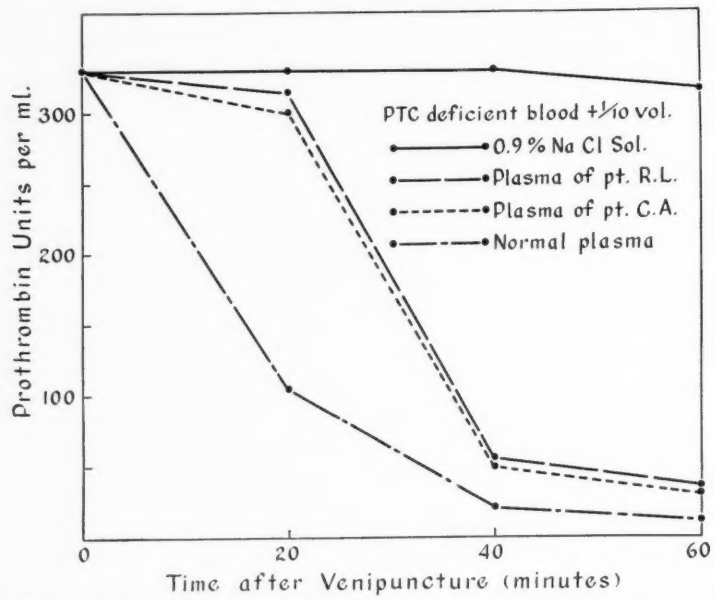

Fig. 3.—Effect of the plasmas of Patients R. L. and C. A. versus normal plasma on the consumption of prothrombin of PTC deficient blood (two-stage method).

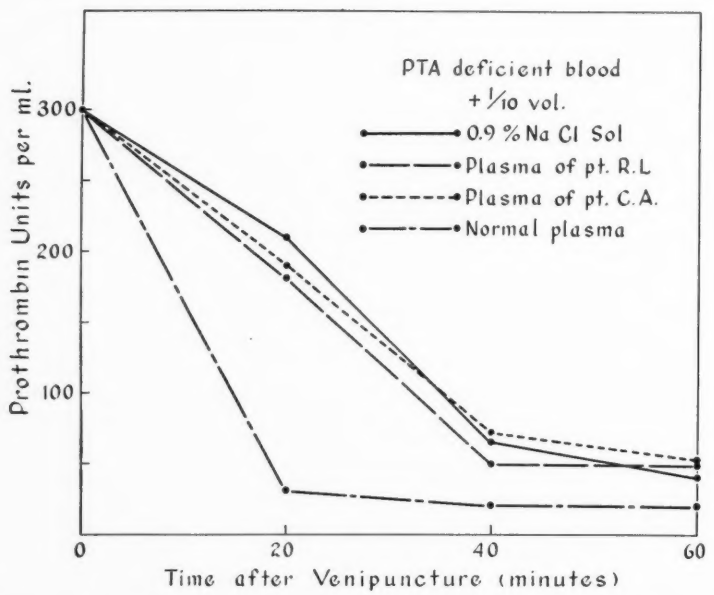

Fig. 4.—Effect of the plasmas of Patients R. L. and C. A. versus normal plasma on the consumption of prothrombin of PTA deficient blood (two-stage method).

tion of PTA deficiency, which was not influenced at all. In the case of AHG deficient and PTC deficient blood, the consumption of prothrombin was improved

TABLE III. EFFECT OF PLASMA OF HAGEMAN'S TRAIT ON THE CLOTTING TIME AND PROTHROMBIN CONSUMPTION (ONE-STAGE METHOD) OF PATIENTS WITH AHG, PTC AND PTA DEFICIENCY

PREPARATION MIXED WITH 1.0 ML. OF BLOOD DEFICIENT IN AHG, PTC, OR PTA	AHG DEFICIENT BLOOD			PTC DEFICIENT BLOOD			PTA DEFICIENT BLOOD		
	CLOTTING TIME IN MINUTES	SERUM PROTHROMBIN TIME IN SEC.		CLOTTING TIME IN MINUTES	SERUM PROTHROMBIN TIME IN SEC.		CLOTTING TIME IN MINUTES	SERUM PROTHROMBIN TIME IN SEC.	
		1 HR.	2 HR.		1 HR.	2 HR.		1 HR.	2 HR.
0.1 ml. 0.9 per cent NaCl solution	45	13.0	14.7	50	15.0	15.0	22	15.0	15.5
0.1 ml. normal plasma	17	30.0	30.4	15	25.2	25.8	16	24.3	26.5
0.1 ml. plasma of patient C. A.	16	19.8	19.8	19	20.0	20.2	19	16.0	16.9
0.1 ml. plasma of patient R. L.	20	16.9	17.8	15	18.6	20.4	20	16.4	16.7

some but not restored to normality. In this second group of experiments, the data obtained with the one-stage method for prothrombin determination (Table III) diverge from two-stage data (Figs. 2, 3, and 4). The one-stage tests indicated that the consumption of prothrombin of AHG deficient and PTC deficient blood was little affected by the plasmas of Patients R. L. and C. A. for a period of two hours after venipuncture. However, the two-stage data showed that there was actually only a delay of prothrombin consumption during the first 40 minutes after venipuncture. The 60-minute values approached the results obtained with the addition of normal plasma. The diversity of data obtained with the two methods remains unexplained.

Even though the first group of experiments demonstrated that the two patients were probably deficient in a substance which was different from AHG, PTC, and PTA, the results of the second group of tests were not so conclusive. Further studies were therefore necessary, especially for the differentiation of the new substance from PTA and from the fourth plasma thromboplastin component of Spaet and associates.[5] Mixing experiments with the blood deficient in the latter factor could not be carried out because of the unavailability of an individual with such a defect.

DETERMINATION OF PHYSICO-CHEMICAL CHARACTERISTICS OF THE NEW FACTOR

The new factor was present in serum and in $BaSO_4$-treated normal plasma (Fig. 5). This separated it distinctly from AHG and PTC[12] but not from PTA and the fourth plasma thromboplastin component of Spaet. The heat resistance characteristics offered more conclusive data. The new factor resisted exposure to 56° C. for 30 minutes and to 60° C. for 15 minutes. It was largely but not completely destroyed after heating to 65° C. for 15 minutes (Fig. 6). The factor of Spaet is heat labile, being destroyed rapidly at 56° C. PTA activity is diminished after exposure to 60° C. for 10 minutes.[4] Hence the new factor was more heat resistant than any of the other plasma precursors of thromboplastin (Table IV). Attempts to precipitate and separate the new substance from normal plasma or serum with various concentrations of a saturated solution of ammonium sulfate were not too successful. Even though the fraction obtained with 25 per cent to 33 per cent saturation gave maximum activity, considerable activity was also present in the 0 to 25 per cent and in the 33 per cent

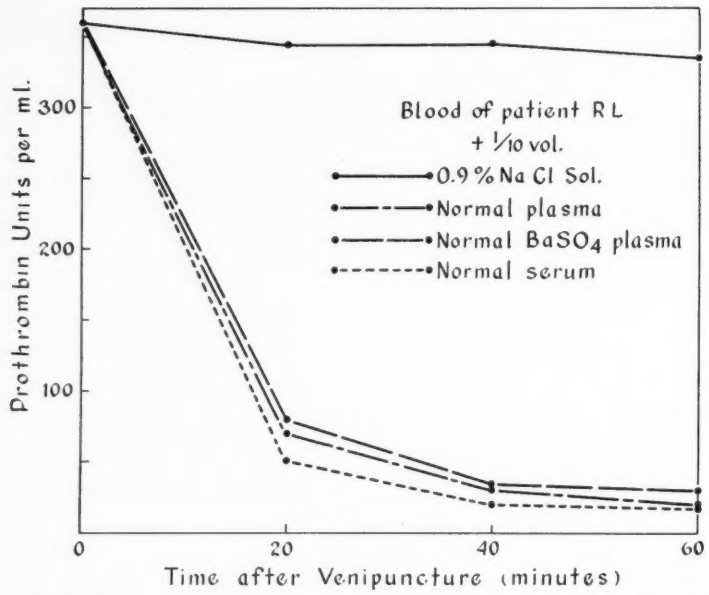

Fig. 5.—Effect of normal plasma, normal serum, and normal $BaSO_4$-treated plasma on the consumption of prothrombin of Patient R. L. (two-stage method).

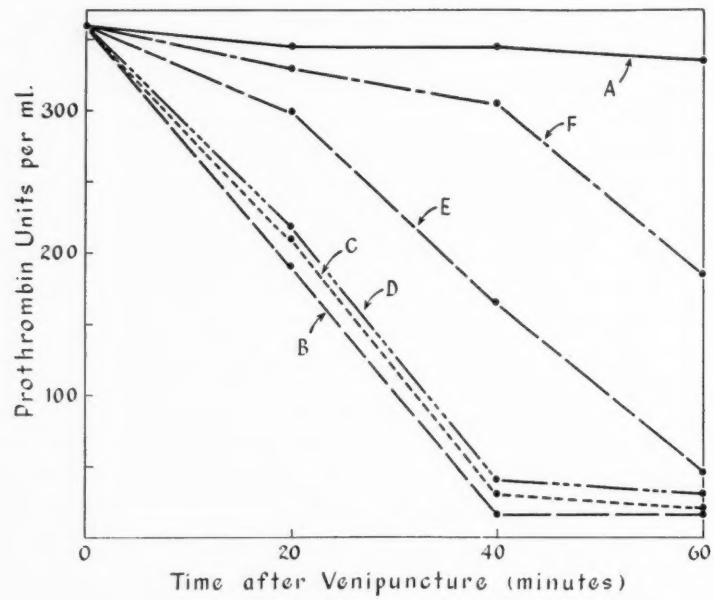

Fig. 6.—Effect of serum exposed to various temperatures for various periods of time on the consumption of prothrombin of Patient R. L. (two-stage method).

A, Blood of Patient R. L. + 1/10 vol. 0.9% NaCl Sol.

B, Blood of Patient R. L. + 1/10 vol. untreated normal serum.

C, Blood of Patient R. L. + 1/10 vol. normal serum heated at 56° C. for 30 minutes.

D, Blood of Patient R. L. + 1/10 vol. normal serum heated at 60° C. for 15 minutes.

E, Blood of Patient R. L. + 1/10 vol. normal serum heated at 60° C. for 30 minutes.

F, Blood of Patient R. L. + 1/10 vol. normal serum heated at 65° C. for 15 minutes.

TABLE IV. PHYSICO-CHEMICAL PROPERTIES OF THE VARIOUS PLASMA THROMBOPLASTIC FACTORS

FRACTION	AHG ACTIVITY	PTC ACTIVITY (3)	PTA ACTIVITY (4)	FOURTH FACTOR OF SPAET (5)	NEW FIFTH FACTOR
Normal plasma	Present	Present	Present	Present	Present
Normal serum	Absent	Present	Present	Present	Present
Normal $BaSO_4$ plasma	Present	Absent	Present	Present	Present
Ammonium sulfate fraction of normal plasma, maximum activity	0-25%	33%-50%	25%-33%	33%-50%	25%-33%
Electrophoretic fraction of normal serum		Between ß and γ globulin	Between ß and γ globulin		Between ß and γ globulin
Normal plasma heated to 56° C. for 30 min.	Present	Absent		Absent	Present
to 60° C. for 15 min.	Diminished	Absent	Diminished	Absent	Present
to 60° C. for 30 min.		Absent		Absent	Diminished
to 60° C. for 15 min.		Absent		Absent	Diminished

to 50 per cent fractions. The electrophoretic separation of the new factor was much easier to achieve. Separate fractions of normal serum were prepared with the technique of Owen and McKenzie[13] with use of paper electrophoresis. Only one fraction, located between the beta and gamma globulins, fully restored the clotting time and the consumption of prothrombin of both patients. The other fractions were ineffective. In this respect the new factor has the same electrophoretic motility as PTA and PTC. The electrophoretic patterns of plasma and serum of both patients were normal. The new factor could not be dialyzed from plasma, serum, or any of the suspensions in normal saline obtained from the precipitates with various concentrations of ammonium sulfate.

In conclusion, it appears that the two patients had a deficiency of a fifth plasma thromboplastin precursor, which has the following characteristics: it is present in normal plasma and serum, cannot be adsorbed on $BaSO_4$, and is not dialyzable. It resists heating to 56° C. for 30 minutes and is largely but not completely destroyed after exposure to 65° C. for 15 minutes. Separation by electrophoresis revealed that it is present in the area between the beta and gamma globulins of normal serum. Its activity was found in all precipitates obtained from normal plasma or serum with 0 to 25 per cent, 25 to 33 per cent, and 33 to 50 per cent saturated ammonium sulfate solution with a maximum in the 25 to 33 per cent fraction.

DISCUSSION

Usually, excessive hemorrhage has prompted studies of abnormal coagulation and hemostasis. With the tests presently available most of the patients with excessive bleeding can usually be diagnosed and classified under one of the categories of congenital or acquired hemorrhagic diseases. The discovery of a severe disturbance of coagulation without hemorrhagic symptoms by Ratnoff constitutes a curiosity and a special challenge. The study of the two patients presented above leaves little doubt that they have the same clinical and laboratory picture reported by Ratnoff in Hageman's trait. The differentiation from other known defects of coagulation in the phase of thromboplastin formation

disclosed several interesting features. The corrective effect of plasmas which were deficient, respectively, in AHG, PTC, and PTA on the abnormality of Hageman's trait was comparable to that of normal plasma. This was not true, however, for the reverse test in which Hageman's trait plasma was tested on AHG, PTC, and PTA deficiency. While in the case of AHG and PTC deficiency, the restorative effect was appreciable, though not complete, the poor consumption of prothrombin in PTA deficiency was not influenced by the plasma of Hageman's trait. These results suggest that in the process of normal coagulation, the new fifth plasma precursor of thromboplastin, which is deficient in Hageman's trait, probably acts in a stage preceding the action of PTC, AHG, and specifically PTA. The mechanism of interaction of the various precursors of thromboplastin is still an enigma, and further studies will be necessary to either prove or disprove this assumption.

A few remarks should be made at this point concerning mixing experiments. In order to obtain consistent and informative data, the plasmas to be mixed with freshly drawn blood should be exposed to the least amount of manipulation and should be used as early as possible after collection. In all experiments reported in this paper, plasma was separated in siliconized equipment and used for mixing experiments within a maximum period of eight hours. Freezing, prolonged storage at 4° C., and exposure to plain glass was purposely avoided, because in our experience these procedures alter the activity of the plasma. It is obvious that these experiments can only be performed when the individuals to be tested are all available on the same day. The experiments presented herein were only made possible through exceptional cooperation of the patients under study.

The differentiation between Hageman's trait and PTA deficiency should not only be based on mixing experiments and the minor differences in physicochemical characteristics between PTA and the fifth precursor of thromboplastin but also on the clinical symptom of hemorrhage. Hemorrhage was a real problem in the five patients with PTA deficiency under observation, while it was absent in the two cases of Hageman's trait. It should be stressed that from a laboratory standpoint the abnormality in PTA deficiency was actually much less marked than the abnormality in Hageman's trait. The paradox of abnormal coagulation with no hemorrhagic symptoms remains unexplained.

The disease is probably congenital. This assumption is based on its occurrence in siblings reported by Ratnoff, on the absence of other metabolic abnormalities, specifically abnormalities of hepatic function, and on the fact that the clotting defect remained unchanged over a period of one and seven years, respectively, in these two patients. The hereditary pattern has not been elucidated. Ratnoff observed the anomaly in both sexes. Unfortunately it has not been possible to study other family members of the presented subjects. The discovery of new cases, which is a matter of chance, may supply the necessary data to establish the heredity of this fascinating disease.

SUMMARY

1. Two patients are presented who had defective coagulation of the blood but had no hemorrhagic symptoms. There was markedly prolonged venous coagulation time, prolonged recalcification clotting time of the plasma, and poor consumption of prothrombin during coagulation. Their abnormality is identical with the newly discovered familial syndrome named Hageman's trait.[2]

2. The differentiation from other conditions with similar laboratory findings grouped under the general term of hemophilia is presented in detail.

3. The new anomaly is caused by a deficiency of a fifth plasma thromboplastin precursor which is present in normal plasma and serum. The factor cannot be adsorbed on $BaSO_4$, is not dialyzable, and resists heating to 56° C. for 30 minutes; exposure to 65° C. for 15 minutes results in appreciable but not complete inactivation. Separation by electrophoresis revealed that the substance is present in the area between the beta and gamma globulins of normal serum. The activity of this factor was found in precipitates obtained from normal plasma and serum with zero to 25 per cent, 25 to 33 per cent, and 33 to 50 per cent saturated ammonium sulfate solution, with a maximum in the 25 to 33 per cent fraction.

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EFFECT OF ADRENAL STEROIDS IN HEREDITARY SPHEROCYTIC ANEMIA

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ADRENAL steroids have been shown to affect the rate of red cell production and destruction in various types of anemia in man.[1-4] In order to further elucidate their manner of action in hemolytic disease, the effect of ACTH and cortisone on red cell turnover in hereditary spherocytic anemia was studied.

MATERIAL AND METHODS

The hematologic response to cortisone and ACTH in one normal individual and in four patients with hereditary spherocytic anemia is reported. The patients had a family history typical of this disease and showed splenomegaly, spherocytosis in their peripheral blood, and an increased osmotic fragility of their erythrocytes. Three patients were studied prior to splenectomy. The fourth patient was studied only after splenectomy. Cortisone was administered, in doses of 400 mg. per day, by mouth over an interval of 15 days in each subject. In one patient, 20 mg. of ACTH, as well as cortisone, was given over a similar period, by intravenous infusion for 8 hours each day.

Reticulocyte preparations were made by exposing blood for 5 minutes to an aqueous solution of brilliant cresyl blue (1 per cent), and counts were made on dried-blood films. Bilirubin was determined by the method of Evelyn and Malloy,[5] stool urobilinogen by the method of Watson,[6] modified slightly,[7] plasma iron turnover according to the method of Huff and associates,[8] serum iron by the method of Kitzes, Elvehjem, and Schuette,[9] osmotic fragility according to Ham,[10] T-1824 blood volume according to Gibson and Evelyn,[11] and with correction for mean body hematocrit and chromium survival as previously described.[12]

RESULTS

Cortisone administered for fifteen days in the normal subject (Fig. 1) was associated with a fall in the hematocrit from 45 to 40 and with a rise in the reticulocytes to 3 per cent. Plasma volume determinations showed the fall in hematocrit to be due to hemodilution. Both findings returned to normal promptly after the cessation of therapy. There was no demonstrable effect on urobilinogen excretion during the experimental period.

The hematologic effects of ACTH and cortisone on Patient J. G., a 69-year-old man with hereditary spherocytic anemia, are shown in Fig. 2. The hematocrit showed little change during treatment. Serum iron rose during the first 10 days and fell thereafter. Reticulocytes during ACTH administration fell from 17.5 to 1.5 per cent, and during cortisone, from 6.0 to 1.5 per cent. The average fecal urobilinogen excretion during the first control period before therapy

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was 2,700 mg. per day, with a maximum of 3,500 and a minimum of 2,000 mg. per day over the 12 days studied. During ACTH administration there was a fall to a minimum of 750 mg. per day, with a rebound after stoppage of therapy to 4,000 mg. per day. A similar fall was observed during cortisone therapy, from

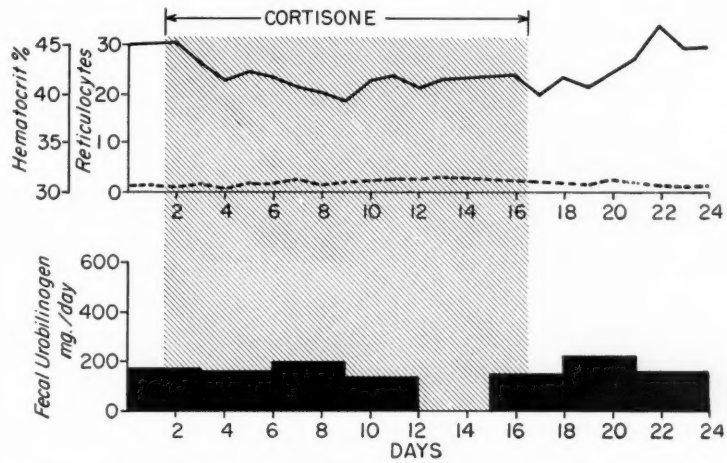

Fig. 1.—Cortisone effect on erythron in normal man. The fall in hematocrit and slight rise in reticulocyte count during therapy reflect the weight gain of 7 kg. and hemodilution produced by cortisone. Stool urobilinogen determination was not performed on days 12 to 15.

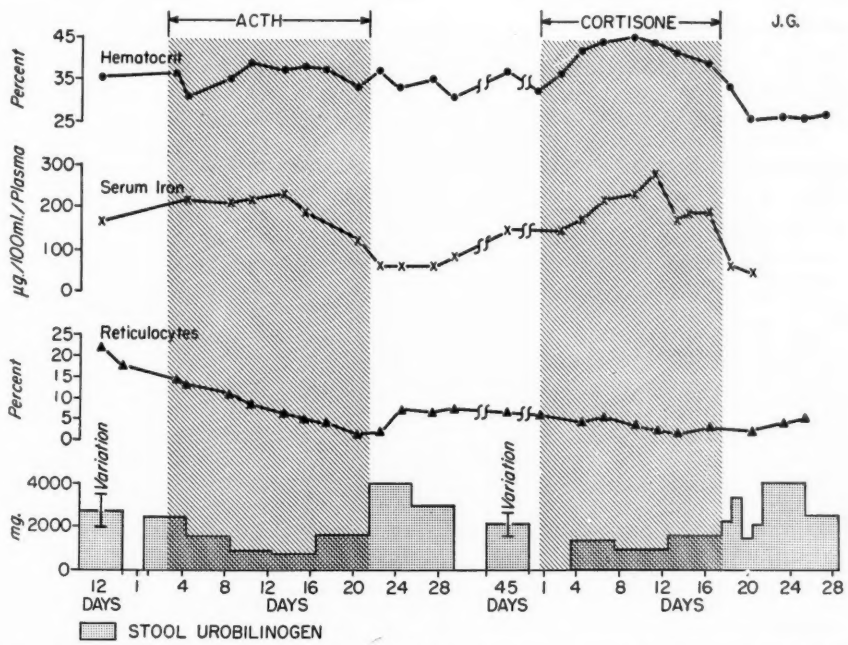

Fig. 2.—Effect of cortisone and ACTH administration in Patient J. G., who had hereditary spherocytic anemia.

an average control value of 2,200 mg. per day to 930 mg. and a rebound to 4,045 mg. per day. Plasma bilirubin levels before cortisone therapy ranged between 0.72 and 1.34 indirect reacting and 1.71 to 4.35 total bilirubin. During therapy

the indirect reacting bilirubin was between 1.24 and 1.4, and the total bilirubin between 3.25 and 5.34. No significant trend was observed. Urine urobilinogen showed a progressive increase during cortisone administration from pretreatment

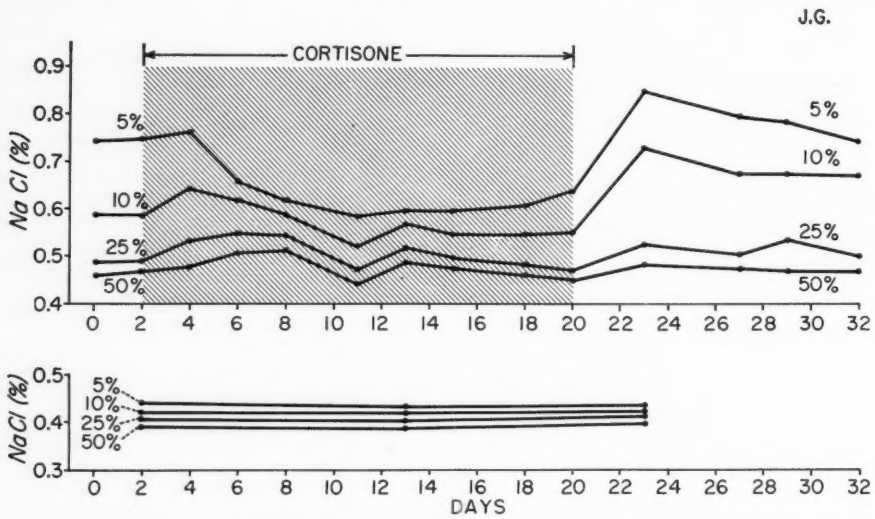

Fig. 3.—Osmotic fragilities are so plotted to indicate the tonicity (% NaCl) at which 5, 10, 25, and 50% hemolysis occurred. The changes in the patient's blood are compared to fragilities run simultaneously on a normal subject (below).

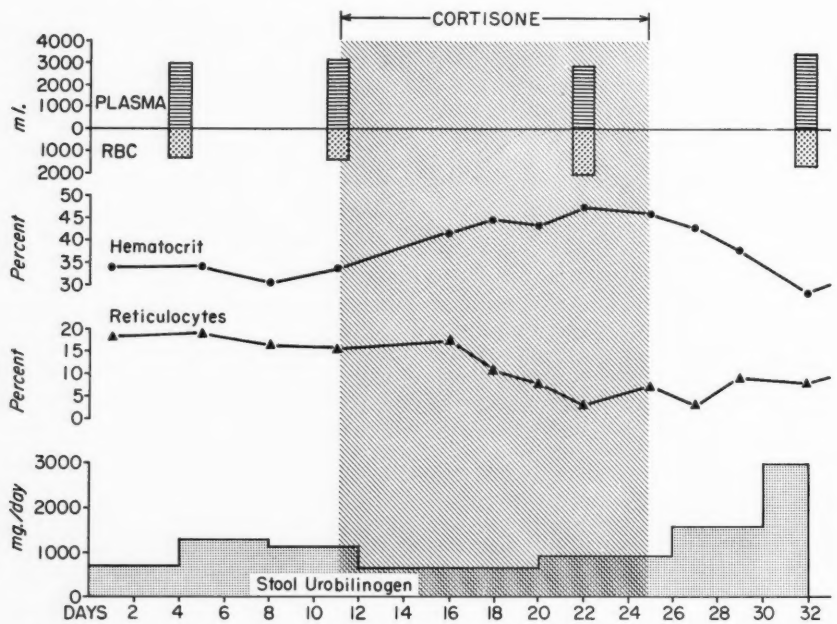

Fig. 4.—Effect of cortisone administration in Patient T. W., who had hereditary spherocytic anemia.

levels of 11 to 29 mg. per day to a maximum of 546 mg. per day on the last day of steroid therapy. Osmotic fragility tests (Fig. 3) indicated an increased resistance of the more fragile population of circulating red cells. The plasma iron

turnover of this patient both before and during cortisone therapy was approximately 250 mg. per day. The amount of radioactive iron appearing in the red cell mass during the control period was 56 per cent of the injected dose, as compared with 22 per cent when determined on the sixth day of cortisone administration. With the employment of the formulae of Huff and associates,[8] it is calculated that approximately 120 mg. of iron per day was being incorporated into the red cell mass initially, while only 60 mg. per day was utilized during cortisone therapy.

The second patient with hereditary spherocytic anemia, T. W., was studied during a control period of 9 days, during 14 days of cortisone administration, and 7 days thereafter (Fig. 4). Changes observed were of a similar nature to those reported in the first subject. The hematocrit rose from 34 to 47 during therapy and fell thereafter to 29 per cent. The serum iron rose from 120 to 197 μg per cent during the first 10 days of therapy and subsequently fell to 94 μg per cent. Reticulocytes fell from a control level of 17 per cent to 3.5 per cent during cortisone. Marrow erythroid cells were 713/1,000 myelocytic cells before therapy and at the sixth day of cortisone therapy were 678/1,000 myelocytic forms (4,000 marrow cells counted). Red cell mass increased from an initial volume of 1,300 ml. to 2,000 ml. on the eleventh day of cortisone administration. Pigment determinations again showed a fall in fecal urobilinogen from 1,050 mg. per day to 650 mg. per day during cortisone, and a poststeroid rise to a maximum of 2,975 mg. in a single day. The urine urobilinogen rose from 1 to 17 mg. per day during cortisone.

P. S., a third patient with hereditary spherocytosis and associated splenomegaly, was studied for a total of 33 days, including 15 days of cortisone administration (Fig. 5). This subject complained of symptoms of weakness and general malaise during the first 4 to 5 days of the control period. Simultaneously his hematocrit fell to a low of 31 but increased to 38 before cortisone was administered. The symptoms during this episode were similar to other attacks which he had had previously and were considered to represent a spontaneous "crisis." During this episode, reticulocytes progressively increased from 19 to 34 per cent, stool urobilinogen was increased to as much as 2,475 mg. per day, and the urine contained up to 114 mg. per day of urobilinogen. The red cell survival as measured by the Cr^{51} technique indicated a half-life of 4 days at this time.

During cortisone administration, the hematocrit continued to rise, while reticulocytes decreased. The stool urobilinogen showed some decrease, with an increase after the discontinuance of steroid therapy. Again the urinary urobilinogen increased during therapy to as much as 107 mg. per day and fell after its discontinuance to less than 25 mg. per day. The Cr^{51} survival studies just preceding steroid administration indicated a half-life of 7 days, which lengthened to a half-life of 10 days during the cortisone period and returned to 7 days thereafter (Fig. 6). Fasting glucose determinations of the blood during steroid administration were unchanged as compared with the control period.

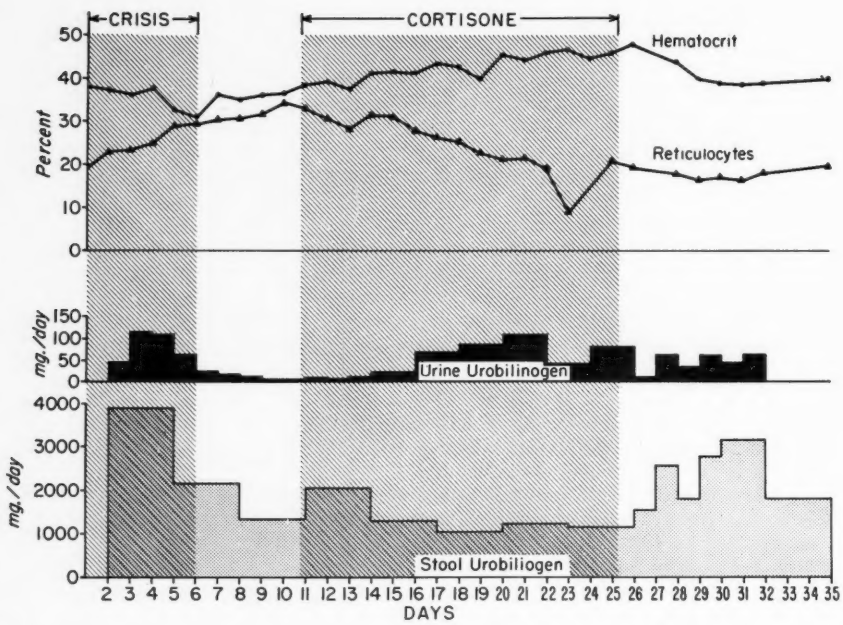

Fig. 5.—Effect of cortisone administration in Patient P. S., who had hereditary spherocytic anemia.

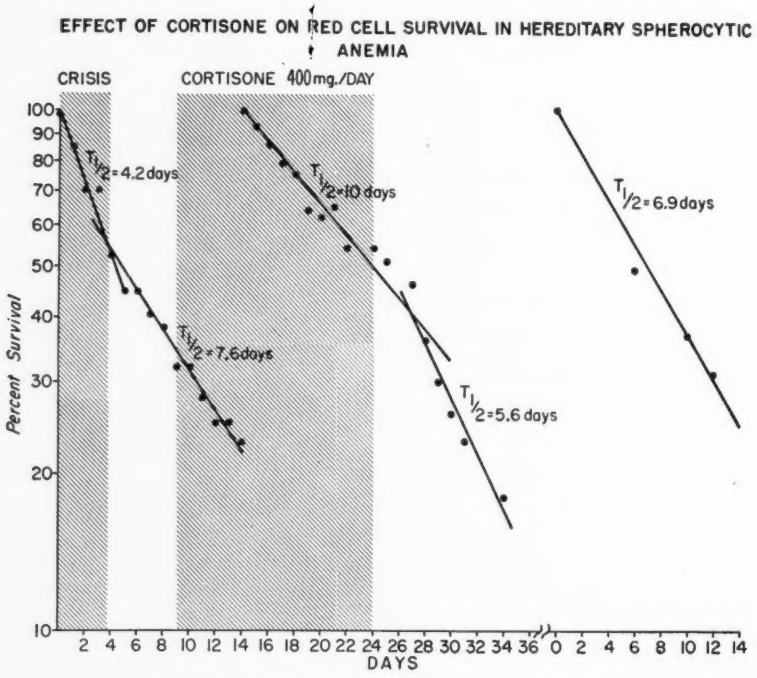

Fig. 6.—Red cell survival studies were performed by the injection of Cr^{51}-tagged red cells obtained from the patient's brother (M. S.), who had the same red cell genotype and had hereditary spherocytic anemia previously treated by splenectomy.

A fourth patient, M. S., who had hereditary spherocytic anemia and had been previously splenectomized, was studied in similar fashion. He showed no significant changes in hematocrit, serum iron, or reticulocytes during therapy. There appeared to be some decrease in fecal urobilinogen, but the overshoot at the end of the steroid therapy was not observed.

DISCUSSION

ACTH, cortisone, and related oxysteroids have been reported to influence red cell production, red cell destruction, and the activities of the spleen. In the hemolytic anemias associated with autosensitization, hematologic improvement has been correlated to some extent with the effect of oxysteroids on circulating antibodies or cell sensitization, as measured by the Coombs test.[13] In other instances where an effect has been produced on anemia, no antibody has been demonstrated, and the mechanism of action is unknown. In an attempt to demonstrate a nonspecific effect of steroids on red cell turnover, patients with hereditary spherocytic anemia have been chosen because of the rapid red cell turnover and the fairly uniform rate of hemolysis from patient to patient. Hereditary spherocytic anemia is characterized by a congenital abnormality in red cell metabolism and structure which renders the red cell susceptible to splenic destruction.[14] The red cell life span is shortened to approximately 10 to 15 per cent of normal,[15] and the level of circulating red cells is relatively constant except for occasional "crises."

The administration of cortisone to three patients with this disorder resulted in an increase in circulating hemoglobin concentration in each instance. The increase was more significant when red cell mass was considered, since cortisone produced expansion of the plasma volume through fluid accumulation. During cortisone therapy there was a significant decrease in fecal urobilinogen in each instance to less than half that found in the control period. This was followed after withdrawal of the steroid by an overshoot to values approximately twice normal. The exact time relationship and magnitude of the changes in fecal urobilinogen to steroid therapy left little doubt of the casual relationship of steroid therapy in these changes. Fifty-seven days of control observations in Patient 1 showed no variations of the magnitude observed with either ACTH or cortisone administration. In one patient, an increased red cell life span, as determined by Cr^{51} during cortisone therapy, provided additional evidence of the decrease in blood destruction.

The reduction in hemolysis became more significant when blood production was examined. There was a progressive fall in reticulocytes in each patient during treatment. There was an associated decrease in the amount of radioiron incorporated into the circulating red cell mass in one patient, indicating again a decrease in red cell production. Thus, there would appear to be not only a decrease in red cell destruction but also a retarded production of red cells. Since the net result was an increase in red cell mass, it seems likely that the slowing in production was a secondary adjustment to the increasing red cell mass.

The spleen is known to be the site of red cell destruction in hereditary spherocytic anemia,[15] and erythrocytes contained within the pulp have a greater

osmotic fragility than those in the peripheral circulation.[14] Following splenectomy, although fragility does not entirely return to normal, the extremely fragile cells present in circulation before splenectomy are no longer found. Likewise, if cells from a patient with this disease who has an intact spleen are transfused into a splenectomized individual, a similar improvement in the fragility pattern occurs.[16] It has been assumed that this increased fragility observed in the spleen is a prelude to cell destruction. In one patient studied here who had the largest spleen, it was possible to show an improvement in osmotic fragility similar to that observed after splenectomy. This would suggest that cortisone interferes with the sphering activity of the spleen and that this reflects a decrease in the destructive capacity of the spleen.

Recent studies have demonstrated an abnormality in carbohydrate metabolism of the red cell in hereditary spherocytic anemia. In vitro studies indicate that in the absence of glucose, these cells undergo sphering and hemolysis with unusual rapidity.[17] The possibility exists that increase in circulating glucose level or some other change in carbohydrate metabolism during cortisone therapy might reduce the rate of blood destruction. Since no significant change in concentration of circulating glucose during steroid administration was found in one patient, there is no supporting evidence that cortisone may have an effect on the metabolic abnormality of hereditary spherocytosis. The fact that increased red cell destruction from splenomegaly of other etiologies is also favorably influenced by cortisone therapy[18] suggests that the effect observed might be some nonspecific action of cortisone on the destructive mechanism of the spleen.

The alteration in the urine urobilinogen observed during cortisone therapy is without obvious explanation. Liver function tests were not altered in one patient, and the blood bilirubin did not change significantly. One can only say that under steroid therapy there may be an increase in urinary urobilinogen occurring without evidence of hepatic impairment and at a time when blood destruction and fecal urobilinogen are decreasing.

In one patient, a spontaneous crisis, accompanied by vague symptoms of malaise and weakness, was observed. At this time the red cell life span was shortened, there was a marked increase in fecal and urinary urobilinogen, and the reticulocyte level increased. These observations are of particular interest in view of the trend in recent years to attribute crisis in hemolytic disease to an impairment in marrow production of red cells.[19, 20] In this instance there was clearly increased blood destruction and an active marrow response to the anemia.

There is no simple explanation apparent for the increase in serum iron during the first week of cortisone therapy. In experimental animals treated with steroids, a fall in serum iron has been observed during steroid administration.[21] In studies of steroid administration in man, no consistent effect of steroids on the serum iron level has been observed.[18]

In the normal individuals studied here and in the patient with spherocytic anemia who had previously been splenectomized, no significant changes in red cell production or destruction were observed. Since the red cell turnover rate of these patients was only one-tenth of that observed in the patients with hemolytic disease, it is assumed that similar changes, if present, would be so small as to be unidentifiable.

CONCLUSION

Cortisone, administered in large amounts, has been shown to temporarily increase the circulating red cell mass in congenital spherocytic anemia. This has been shown to be due to a decrease in erythrocyte destruction, which is resumed at an increased rate after cortisone is discontinued. Blood production is temporarily suppressed, secondarily to the increase in the red cell mass. These observations are believed to represent a nonspecific effect of cortisone on red cell destruction by the spleen.

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OVER-ALL COAGULABILITY IN PATIENTS RECEIVING DICUMAROL THERAPY WITH EMPHASIS ON ACUTE MYOCARDIAL INFARCTION

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WITH THE TECHNICAL ASSISTANCE OF ESTHER GENDELMAN, B.S.

THE one-stage prothrombin time[1] has been generally accepted as the coagulation test of choice in the control of anticoagulant therapy with dicumarol and related compounds. While this test provides an indicator of combined changes in prothrombin and proconvertin concentration, it measures only one part of the clotting mechanism, because the addition of an excessive amount of tissue thromboplastin masks any change which may occur in the platelets or plasma thromboplastin factors[2]: antihemophilic globulin (AHG), plasma thromboplastin component (PTC), and plasma thromboplastin antecedent (PTA). An undetected deficiency in any of these factors superimposed upon the deficiencies reflected by the prothrombin time could lead to hemorrhagic complications. On the other hand, an increased coagulability of the blood, as has been demonstrated following acute myocardial infarction,[3, 4] may require lower prothrombin levels in the achievement of a therapeutic result.

The measurement of "over-all" coagulability during dicumarol therapy has received some attention. Measurement of the clotting time in glass tubes has proved relatively insensitive.[5] Moloney and associates[6] and Margulies and Barker[7] were able to detect changes more sensitively by the use of silicone-lined tubes, but this procedure has proved too variable, and the preparation of glassware is too time-consuming. In our previous studies, the use of the heparin clotting time, a simplified modification of the heparin tolerance test, seemed to be of value in controlling dicumarol therapy.[4] Soulier and le Bolloc'h[8] independently performed similar studies. This paper presents further coagulation studies in patients with myocardial infarction, rheumatic heart disease, and thrombophlebitis treated with dicumarol.

METHOD

More than 290 serial coagulation studies were performed upon 21 patients treated with dicumarol and listed in Table I. The following tests were performed by methods previously described[2, 4, 9]: coagulation time measured both at 37° C. (CT) and at room temperature; heparin clotting time (HCT), with addition of 4 γ heparin to 1 ml. of blood, performed at 37° C.; and a variant of the heparin clotting time, with addition of 1 γ heparin to 1 ml. blood, performed at room temperature; and prothrombin time (PT) performed by Quick's method,[1] with use of rabbit brain thromboplastin. Control values for these tests obtained in normal subjects are shown in Table II. In certain instances platelet count, recalcified

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plasma clotting times, and serum prothrombin times were performed. For purposes of analysis, the absolute ratio of the 4 γ heparin clotting time (HCT): prothrombin time (PT) was calculated and designated as HCT:PT ratio.

TABLE I. CLINICAL DATA IN PATIENTS TREATED WITH DICUMAROL

SUBJECT	SEX	AGE (YEAR)	DIAGNOSIS*	DURATION OF STUDY (WEEKS)	NUMBER OF STUDIES	TIME AFTER ONSET, STUDY BEGUN (DAYS)	REMARKS
S. S.	F	60	AMI (ant. wall)	5	20	3	Chart, Fig. 5
J. F.	M	65	AMI (diffuse)	3	16	6	Chart, Fig. 6
H. C.	M	65	AMI (ant. wall)	1½	6	2	Melena, marked diverticulosis of colon
S. K.	M	46	AMI (ant. wall, extensive)	4½	20	6	Chart, Fig. 7
J. S.	M	54	AMI (ant. wall)	4½	19	5	Thrombophlebitis, (?) pulmonary infarct, 14 days after AMI
A. B.	F	62	AMI (ant. wall)	3½	18	5	Chart, Fig. 8
S. A.	F	60	AMI (ant. wall)	3½	14	24	
M. J.	F	42	AMI (post. wall)	3½	8	31	
S. B.	F	51	AMI (post. wall)	3	10	21	
M. J.	M	52	AMI (ant. wall)	1	5	45	
I. P.	F	60	AMI	4	21	14	Chart, Fig. 9
M. S.	F	68	(?) AMI (post. wall)	1	4	3	AMI 3 mo. ago
R. B.	F	58	Angina, myoc. damage	2	6	17	Hypertension 15 yr.
J. G.	F	32	PP thrombophleb.	1	7	5 PP	
L. B.	F	28	PP thrombophleb.	2	9	4 PP	
F. K.	F	22	PP thrombophleb.	1	4	4 PP	
A. C.	F	39	RHD, MS	9½	23		Ambulatory and hospital
R. R.	F	43	RHD, MS	11	21		Ambulatory and hospital
L. R.	F	38	Chr. thrombophleb.	11	20		Ambulatory
S. S.	M	62	Chr. thrombophleb.	11½	19		Amb., chart, Fig. 10
V. F.	M	59	Acute thrombophleb.	7	24	1	

*AMI, Acute myocardial infarction; PP, post-partum; RHD, rheumatic heart disease; MS, mitral stenosis.

TABLE II. COAGULATION TESTS IN NORMAL AND DICUMAROL-TREATED SUBJECTS

TEST	NORMAL, UNTREATED SUBJECTS				DICUMAROL TREATED SUBJECTS							
	NO.	MEAN	MEAN ± 2 S.D.	RANGE	NO.	LOW VALUE NO.	LOW VALUE %	NORMAL VALUE NO.	NORMAL VALUE %	HIGH VALUE NO.	HIGH VALUE %
Clotting time at room temp. (min.)	98	12.8	6.2-19.4	6-23	297			228	74	69	26
Clotting time at 37° C. (CT) (min.)	98	8.2	5.2-11.2	4-12	297			201	68	96	32
1 γ heparin clotting time (min.)	95	22.5	11.7-33.3	13-37	290			175	60	115	40
4 γ heparin clotting time (HCT) (min.)	99	26.4	18.4-34.4	17-37	296	6	2	107	36	183	62
Prothrombin time (sec.)	78	13.6	11.5-15.6		310			26	8	284	92

RESULTS

As plotted in Figs. 1 to 3, the correlation of the prothrombin time with the clotting time, heparin clotting time, and recalcified plasma clotting time was poor. Although there was a rough direct relationship in each case, there was a wide range of values at any one prothrombin level. This was most prominent in the case of the heparin clotting time. The distribution of values for each

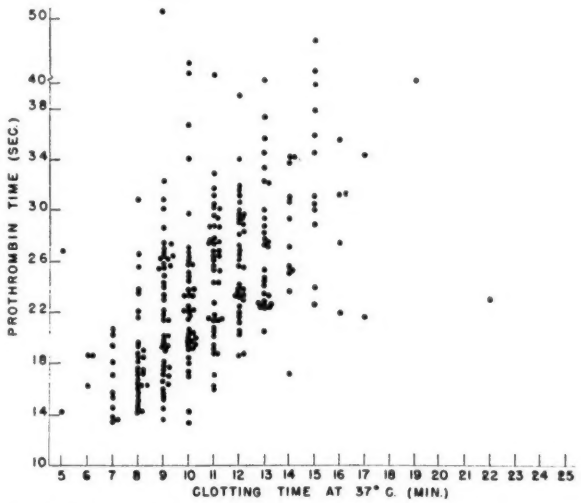

Fig. 1.—Relation of the clotting time to the prothrombin time.

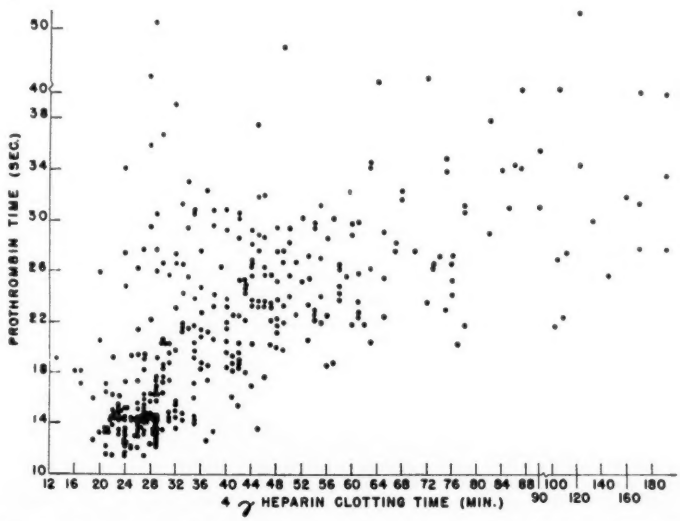

Fig. 2.—Relation of the heparin clotting time to the prothrombin time.

test in the dicumarol-treated patients revealed that subnormal values were noted only for the 4 γ heparin clotting time (Table II). Values above the normal range were noted most frequently in the 4 γ heparin clotting time (62 per cent), followed by the 1 γ heparin clotting time, 37° C. clotting time, and room temperature clotting time.

The HCT : PT ratio was used as an index for further analysis of the results. The index was between 1.5 to 2.5 for control subjects not receiving dicumarol. Values below 1 indicated over-all hypercoagulability and those above 2.5 indicated hypocoagulability in patients treated with dicumarol. The distribution of values for various groups of patients on Dicumarol therapy are shown in Table III. Patients studied less than 3 weeks after the onset of acute myocardial infarction showed an increased incidence (24 per cent) of ratios below 1, indicative of over-all hypercoagulability. It is of interest that 15 per cent of the values were above 2.5, indicating hypocoagulability. Values obtained more than 3 weeks after infarction revealed no evidence of hypercoagulability (ratio below 1), while 23 per cent of the values above 2.5 indicataed slightly increased incidence of hypocoagulability. The distribution of values in the post-partum, rheumatic heart disease, and thrombophlebitic groups revealed occasional hypercoagulability and hypocoagulability in less than 15 per cent of the cases.

TABLE III. RATIO OF HEPARIN CLOTTING TIME TO PROTHROMBIN TIME (HCT:PT) IN PATIENTS TREATED WITH DICUMAROL AND IN RELATION TO BLEEDING MANIFESTATIONS

		HCT:PT RATIO							
		< 1 (HYPER-COAGULA-BILITY)		1 TO 1.5		1.5 TO 2.5		> 2.5 (HYPO-COAGULA-BILITY)	
DISEASE	NO.								
Control-no dicumarol	60	0		1	2%	59	98%	0	
Acute myoc. inf.									
< 3 wk. after onset	59	14	24%	10	17%	26	44%	9	15%
> 3 wk. after onset	101	0		12	12%	66	66%	23	23%
Post partum	20	2	10%	7	35%	10	50%	1	55%
Rheum. heart dis.	40	2	5%	12	30%	20	50%	6	15%
Thrombophlebitis	70	0		8	11%	53	76%	9	13%
Bleeding manifestations									
None		3		4		7		2	
Slight to moderate				1		5		5	
Marked				1				3	

The coagulation results of the two extreme groups of patients, Group A with ratios less than 1.0 and Group B with ratios above 2.5, are compared in Fig. 4. A marked difference was noted between the two groups in the 4 γ heparin clotting times at room temperature and particularly at 37° C. The clotting times showed minimal differences, and the prothrombin times revealed no difference between the two groups.

Serial studies in individual cases are presented in Figs. 5 to 10. Hypercoagulability, as reflected by a HCT : PT ratio, was found in 3 of 6 patients less than 3 weeks after onset of myocardial infarction. As shown in Figs. 5, 6, and 7, the hypercoagulability followed no consistent pattern. Patient S.K. is of particular interest. On two successive days (6 and 7 days after onset) definite hypocoagulability was noted, and an episode of gross hematuria occurred. At this time, the prothrombin time was 23 seconds and the 4 γ heparin clotting time was 75 minutes. On the following day, a sudden hypercoagulability developed with a prothrombin time of 18 seconds and HCT of 17 min-

utes, a value significantly below the normal range. The urine became clear. The hypercoagulable period lasted for about 6 days, after which the coagulability was in the normal range. Fig. 8 presents the data in the case of

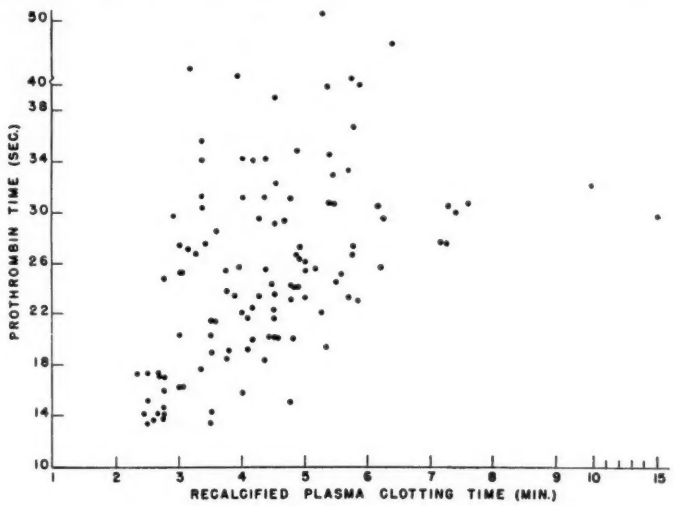

Fig. 3.—Relation of the recalcified plasma clotting time to the prothrombin time.

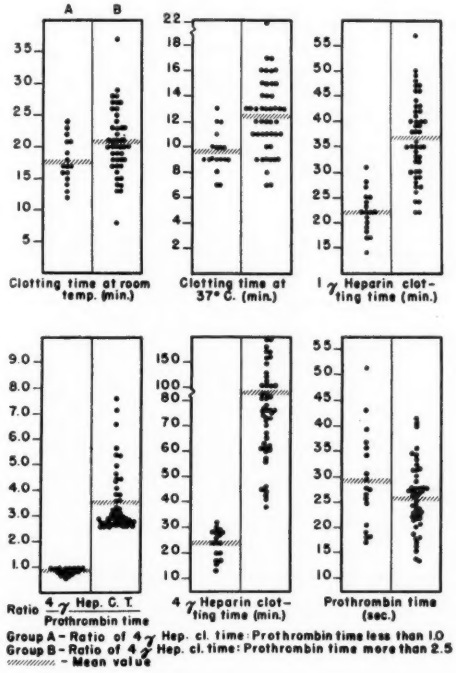
A
B
Clotting time at room temp. (min.)
Clotting time at 37° C. (min.)
1 γ Heparin clotting time (min.)
Ratio 4 γ Hep. C. T. / Prothrombin time
4 γ Heparin clotting time (min.)
Prothrombin time (sec.)
Group A - Ratio of 4 γ Hep. cl. time : Prothrombin time less than 1.0
Group B - Ratio of 4 γ Hep. cl. time : Prothrombin time more than 2.5
- Mean value

Fig. 4.—Distribution of values of the various clotting tests comparing Group A (HCT:PT ratio less than 1) with Group B (HCT:PT ratio above 2.5).

Patient A. B. whose coagulation occasionally fluctuated into the hypocoagulable range but was never accelerated. Patient I. P. (Fig. 9), with a recent myo-

cardial infarction, revealed normal coagulability except for a sudden spike of hypocoagulability 25 days after the onset of Dicumarol therapy. At this time, the urine contained many red cells by microscopic examination, whereas no red cells were found on two other occasions during normal coagulability. Fig. 10

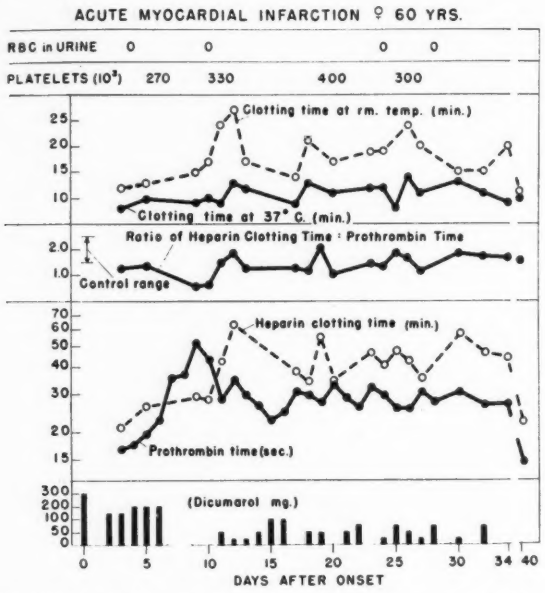
ACUTE MYOCARDIAL INFARCTION ♀ 60 YRS.
RBC in URINE 0 0 0 0
PLATELETS (10³) 270 330 400 300
Clotting time at rm. temp. (min.)
Clotting time at 37° C. (min.)
Ratio of Heparin Clotting Time : Prothrombin Time
Control range
Heparin clotting time (min.)
Prothrombin time (sec.)
(Dicumarol mg.)
DAYS AFTER ONSET

Fig. 5.—Serial coagulation studies performed on Patient S. S., acute myocardial infarction.

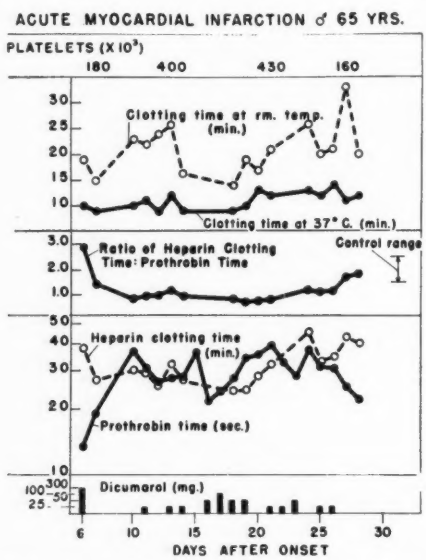
ACUTE MYOCARDIAL INFARCTION ♂ 65 YRS.
PLATELETS (X 10³)
180 400 430 160
Clotting time at rm. temp. (min.)
Clotting time at 37° C. (min.)
Ratio of Heparin Clotting Time: Prothrobin Time
Control range
Heparin clotting time (min.)
Prothrobin time (sec.)
Dicumarol (mg.)
DAYS AFTER ONSET

Fig. 6.—Serial coagulation studies performed on Patient J. F., acute myocardial infarction.

presents the data of a patient with chronic thrombophlebitis treated on an ambulatory basis. Coagulability was generally normal, with a close parallel between the HCT and PT, except for two spikes of hypocoagulability.

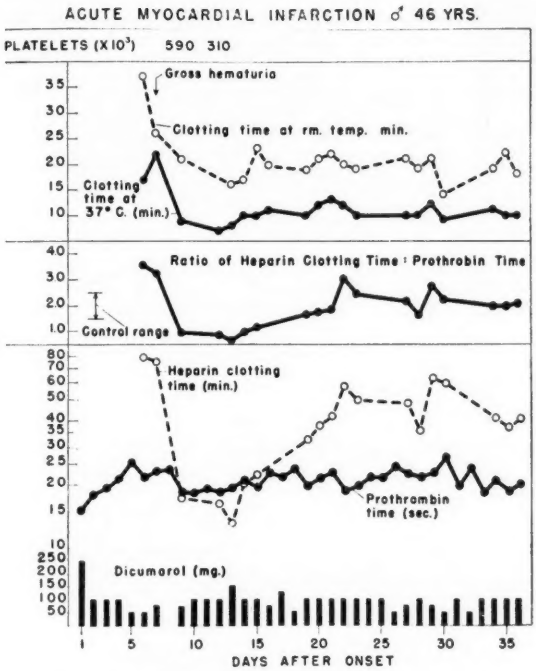
ACUTE MYOCARDIAL INFARCTION ♂ 46 YRS.
PLATELETS (X10³) 590 310
Gross hematuria
Clotting time at rm. temp. min.
Clotting time at 37° C. (min.)
Ratio of Heparin Clotting Time : Prothrobin Time
Control range
Heparin clotting time (min.)
Prothrombin time (sec.)
Dicumarol (mg.)
DAYS AFTER ONSET

Fig. 7.—Serial coagulation studies performed on Patient S. K., acute myocardial infarction. Note the sudden change from hypocoagulability to hypercoagulability detected by the heparin clotting time in the 7- to 10-day period after onset. During this time the prothrombin time showed little variation.

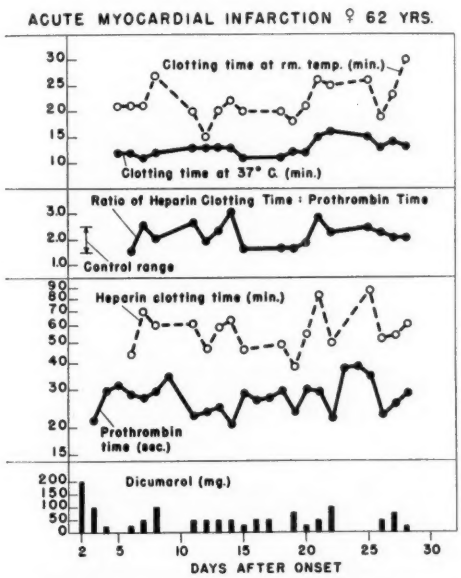
ACUTE MYOCARDIAL INFARCTION ♀ 62 YRS.
Clotting time at rm. temp. (min.)
Clotting time at 37° C. (min.)
Ratio of Heparin Clotting Time : Prothrombin Time
Control range
Heparin clotting time (min.)
Prothrombin time (sec.)
Dicumarol (mg.)
DAYS AFTER ONSET

Fig. 8.—Serial coagulation studies performed on Patient A. B., acute myocardial infarction.

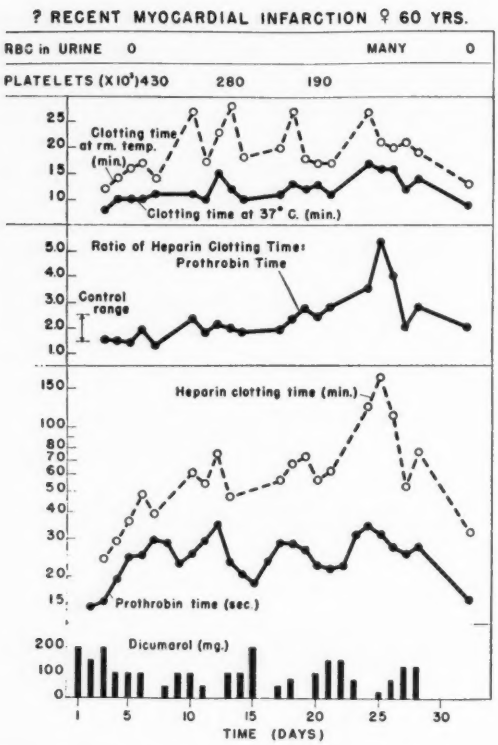

Fig. 9.—Serial coagulation studies performed on Patient I. P., recent myocardial infarction.

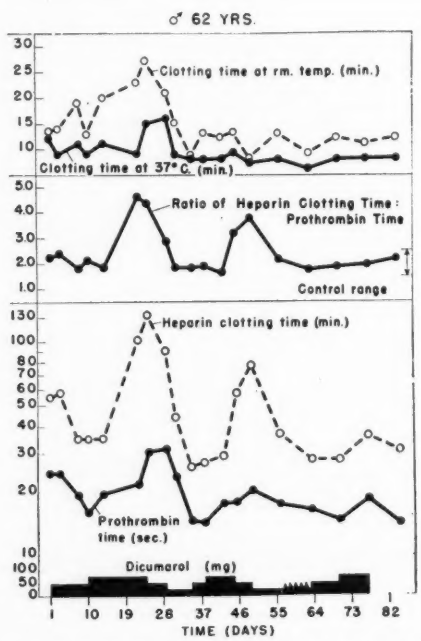

Fig. 10.—Serial coagulation studies performed on Patient S. S., treated on an ambulatory basis for chronic thrombophlebitis.

Observations of the relation of abnormal bleeding to the degree of coagulabilitiy are summarized in Table III. Although no definite conclusions can be drawn, severe hemorrhagic complications were more prone to develop during hypocoagulable periods. In the present study, bleeding was manifested chiefly by hematuria, either gross or microscopic, epistaxis, or hematoma following injections. Patient H. C. developed melena four days after the onset of therapy. His PT of 31 seconds and HCT of 40 minutes indicated a normal coagulability index, but large bowel x-rays revealed marked diverticulosis of the colon as the probable source of bleeding.

One thromboembolic episode was noted during the study: Patient J. S. developed thrombophlebitis of the left calf and a probable pulmonary infarction 14 days after the onset of acute myocardial infarction. During this period, his coagulability was in the normal range with PT 23 seconds and HCT 49 minutes.

DISCUSSION

These studies substantiate previous observations that the prothrombin time is an inadequate measurement of over-all coagulability.[4, 8] A poor correlation was found between the PT and other tests: CT, HCT, and plasma clotting time. Comparison of various tests indicated that the 4 γ heparin clotting time, used in conjunction with the prothrombin time, provided a sensitive and easily obtained measurement of total coagulability. The coagulation time performed in glass tubes either at room temperature or at 37° C. did not provide adequate sensitivity for the detection of either accelerated or retarded coagulability.

The exact mechanism of the coagulation changes which would explain the discrepancies between the PT and HCT are not known. Many workers have obtained evidence that changes in the prothrombin time following the administration of Dicumarol and related substances are produced by alterations in more than one clotting factor, particularly prothrombin and proconvertin.[10-18] The relative changes in these two clotting factors may produce variations in over-all coagulability and the HCT at any given prothrombin time. Our studies on a patient with hereditary hypoproconvertinemia, with a prothrombin time of 30 seconds, have revealed a consistently normal heparin clotting time.[19] Therefore diminution particularly of prothrombin following dicumarol may be an important factor in hypocoagulability. There are suggestions that bleeding may be more likely to occur when the prothrombin concentration as determined by the two-stage method[20] falls below 10 per cent.[13, 21]

It is possible that Dicumarol may affect clotting factors other than prothrombin and proconvertin. There are indications that plasma thromboplastin factors, particularly plasma thromboplastin component (PTC)[22] and Factor X[23] are reduced by Dicumarol therapy. Our studies have also suggested that Dicumarol may depress PTC as shown in an experiment listed in Table IV. Deficiency in plasma thromboplastin factors would accentuate over-all hypocoagulability by further prolonging the HCT[2] without influencing the PT.

TABLE IV. ASSAY OF PTC IN DICUMAROL PLASMA

ADDED TO 1 ML. PTC DEFICIENT BLOOD	CLOTTING TIME (MIN.)	SERUM PROTHROMBIN TIME (SEC.)		DEGREE OF CORRECTION
		AT 2 HR.	AT 48 HR.	
0.1 ml. 0.85% NaCl	28	9	19	
0.05 ml. Dicumarol plasma*	13	13	23	Slight
0.1 ml. Dicumarol plasma*	12	13		Slight
0.05 ml. Control plasma	12	19	33	Moderate
0.1 ml. Control plasma	12	21		Moderate

*Prothrombin time 24 sec., control 15 sec. Patient had received Dicumarol for 1½ months.

On the other hand, the patients' diseases may affect clotting factors, platelets, or clotting equilibrium in a manner not reflected in the prothrombin time but detectable by the HCT. This is exemplified by the hypercoagulability previously detected by the HCT in acute myocardial infarction. The mechanism of this finding is not known. It may be partially related to increased stress and adrenal function after infarction.[24] The hypercoagulability was not eliminated by dicumarol therapy in 3 of 5 patients studied within 3 weeks after the onset. This raises the question of the value of Dicumarol in controlling the hypercoagulability. The patients with acute myocardial infarction also gave evidence of hypocoagulability, which could predispose to hemorrhage in the presence of anticoagulant therapy. Generally, in the six weeks following myocardial infarction, there appears to be an instability of the clotting mechanism with fluctuations between hypocoagulability and hypercoagulability, which merit more emphasis in the use and evaluation of anticoagulant therapy.[25] Similar findings have been reported by Beaumont, Chevalier, and Lenegre,[26] who have proposed 3 phases following an attack of acute myocardial infarction: (1) constant period of hypercoagulability in the first 24 to 48 hours; (2) spontaneous, secondary hypocoagulability lasting about 7 days in 81 per cent of 70 cases; (3) later period of hypercoagulability starting on the eighth or tenth day and lasting a few weeks to possibly several months.

Peel[27] used the heparin clotting time as the indicator for anticoagulant therapy in 88 cases of acute myocardial infarction. Forty-eight patients (55 per cent) revealed rapid heparin clotting times and were given anticoagulant therapy, which was withheld from 40 patients with a normal heparin clotting time. It is remarkable that in this latter group complications were less frequent, mortality was lower, and second attacks within six months were fewer in number (3 per cent compared with 13 per cent).

These studies emphasize that definition of a "therapeutic range" in anticoagulant therapy with Dicumarol and similar drugs cannot be based solely on the prothrombin time but must take into account over-all coagulability, which can be affected both by the anticoagulant and the patient's disease. At the present time, knowledge is insufficient to state the optimum level of coagulability to prevent abnormal bleeding and suppress abnormal intravascular clotting. It is generally accepted that in an intact vascular system, bleeding will not occur even at high levels of hypocoagulability. However, the presence

of a lesion or vascular damage may predispose to bleeding at any level. Avoidance of extremes of over-all hypocoagulability and hypercoagulability may help in the reduction of both hemorrhagic and thrombotic complications.

SUMMARY

1. Serial coagulation studies were performed on 21 patients receiving Dicumarol. From the ratio of the heparin clotting time (HCT) to the one-stage prothrombin time (PT), an index of over-all coagulability was obtained.

2. The PT showed a rough correlation with other clotting tests which reflect over-all coagulability, particularly the heparin clotting time. This was attributed to limitations in the PT which measures only one part of the clotting mechanism and does not reflect changes in thromboplastic factors (platelets, AHG, PTC, PTA, etc.). These factors may be affected by either Dicumarol (particularly PTC) or the patient's disease.

3. Studies on patients with acute myocardial infarction revealed hypercoagulability fluctuating with hypocoagulability, superimposed upon the Dicumarol effects. Hemorrhagic manifestations seemed more closely related to over-all hypocoagulabilitiy than to the prothrombin time alone.

4. The significance of over-all coagulability in the control of anticoagulant therapy with Dicumarol and related drugs is discussed, particularly in relation to acute myocardial infarction. These studies suggested that the prothrombin time alone may be inadequate as the basis of a therapeutic range and in the control of therapy with Dicumarol and related compounds.

I wish to thank Drs. A. Fishberg, S. Rosenfeld, and H. Vessell for providing some of the patients included in this study.

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LABORATORY METHODS

EVALUATION OF A RAPID MICROMETHOD FOR THE DETERMINATION OF SERUM CALCIUM

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INTRODUCTION

THERE is an obvious need for a rapid and accurate method for the determination of serum calcium, particularly as an aid in the diagnosis and management of patients in convulsive states.

The most popular method for the determination of calcium in body fluids involves its precipitation and isolation as the oxalate.[1] The earliest technique for serum calcium determination using this principle was that of Pribram in 1871,[2] in which ammonium exalate was used for the direct precipitation of calcium from serum diluted with water; deWard[3] and Clark[4, 5] applied this oxalate technique to the dissolved ash of serum; Lyman,[6] and Halverson and Bergeim[7] used a protein-free filtrate, to which the ammonium oxalate was added. From these three techniques, namely, (a) direct precipitation, (b) ashing, and (c) deproteinization, stem many of our modern serum calcium determinations through oxalate precipitation. Titration of the oxalate ion with potassium permanganate, as described by Kramer and Tisdall,[8] and later modified by Clark and Collip,[9] is usually the method of choice in present day routine clinical work.[10] Titration with ceric sulfate, a more stable substitute for potassium permanganate, is gaining in popularity.[11] Iodometric and colorimetric modifications of these techniques have been introduced.[12]

Conversion of calcium oxalate to the carbonate or to the oxide, by controlled heating and subsequent titration with acid, has been used as an acidimetric modification.[13, 14] By the gasometric techniques of Van Slyke and Sendroy,[15] the oxalate is converted to carbon dioxide, which is measured in the Van Slyke-Neill manometric gas analysis apparatus. Calcium in this way can be measured with an accuracy of 0.5 to 1.0 per cent. The time required for all oxalate precipitation methods is a serious disadvantage in clinical work, where prompt results may be needed.

Calcium can also be precipitated as the phosphate, being measured colorimetrically as the phosphate by the method of Roe and Kahn,[16] with use of the

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principle of Fiske and Subbarow[17] for the determination of phosphorus. This method, as in the oxalate precipitation, encourages overnight standing to insure complete precipitation.

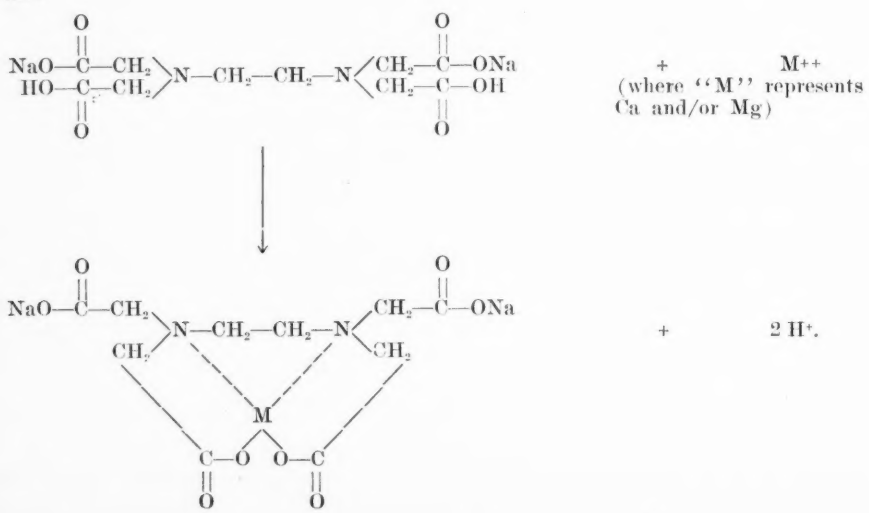
The flame photometer has provided a rapid and accurate means for the quantitative determination of sodium and potassium in serum. Despite claims for its use in the determination of serum calcium,[18] and also magnesium, its dependability in this role, for routine clinical use, has yet to be generally accepted. Spectographic analysis, although impractical for routine clinical work, has furnished accurate results which can be used as checks on other methods.[19] Turbidimetric methods have been proposed for the rapid determination of serum calcium, based on turbidities formed with either solutions of ammonium oxalate or potassium oleate.[20, 21]

The present method is based on those principles used by Schwartzenbach and Ackerman[22] for the determination of water hardness. Aqueous solutions of certain organic dyes form soluble colored complexes with many metals, the alkaline earths in particular. At definite hydrogen ion concentrations the original color of the dye is restored by the addition of sufficient disodium dihydrogen ethylenediaminetetraacetate. This compound is also known as "SED"[23] and "Disodium Dihydrogen Versenate."[24]

Buckley[25] utilized this principle for the determination of calcium and magnesium in body fluids on the macro scale. A separate dye was used for each of the two metals: "Murexide" (ammonium purpurate) for calcium, and the azo dye Eriochrome Black T (Eriochrome Schwartz T) for magnesium, together with a precisely prepared and controlled buffer mixture of ammonium chloride and ammonium hydroxide.

Sobel and Hanok[23] introduced an ultramicro application of this principle, using 0.02 ml. of serum and intricate ultramicro apparatus. These workers used only one dye, Eriochrome Black T, as an indicator for both metals and an easily prepared buffer, ethanolamine.

Alkaline earth metals, as calcium and magnesium in serum, rapidly form chelate complexes with the disodium dihydrogen ethylenediaminetetraacetate as follows:



This chelation of both calcium and magnesium occurs optimally at a pH range of 10 to 11. The precise pH of 10.85, used by Buckley, was replaced in this investigation by the satisfactory pH range of 10.0 to 10.5, suggested by Sobel. A methanol solution of the blue dye Eriochrome Black T, also suggested by Sobel, is used in the proposed method, forming a wine-red-colored complex with calcium and magnesium. The original blue color of the buffered dye-indicator is gradually restored by titration with an equeous solution of SED, which forms the chelate complex, thus removing the metals from combination with the dye. Reversion to the original blue color of the buffered dye-indicator is taken as the end point of the titration.

A similar titration is performed on an equal volume of standard calcium solution. From the SED titration values of both standards and serum and from the strength of the calcium standard used, the total concentration of calcium and magnesium in the serium is calculated. The calcium concentration may then be readily estimated by subtracting a mean serum magnesium value. More precise calcium values may be obtained by accurately determining serum magnesium, and subtracting this value from the total. In like manner, precise magnesium values may be obtained by actually determining serum calcium and subtracting this from the initial sum of calcium plus magnesium.

The aim of this investigation was to evaluate a method for serum calcium which, while maintaining satisfactory accuracy, met the clinicians' demand for speed of determination and still remained simple enough for the average laboratory technician to carry out. The techniques of Buckley, and of Sobel and Hanok, as previously discussed, appeared to be a good starting point in the development of such a method. Accordingly, their principles have been followed, with the adaption of them to the estimation of calcium in 0.1 ml. of serum.

Since the method used gives the sum of calcium and magnesium, the calcium being estimated by subtracting a mean magnesium value, the determination of magnesium has been necessary in this investigation as a control measure.

The determination of serum magnesium, while not yet as popular as that of serum calcium, has been based on its precipitation as magnesium ammonium phosphate. This is accomplished in the methods of Kramer and Tisdall,[8] and of Briggs,[26] with subsequent gravimetric determination of the magnesium ammonium phosphate or its colorimetric determination as the phosphate by the method of Fiske and Subbarow.[17] Precipitation as the 8-hydroxyquinolinolate, with subsequent titrametric or colorimetric determination, has been used.[27]

More promising are methods based on observations by Kolthoff[28, 29] that when magnesium is precipitated as the hydroxide by sodium hydroxide in the presence of an acridine-sulfo-dye, "Titan Yellow" (also known as "Clayton Yellow"), the color of the dye is changed to red. This principle has been the basis for several colorimetric methods for the determination of magnesium in serum and urine. Most notable is that of Garner,[30] with many modifications by other investigators.[31] The most recent contribution using this principle is that of Orange and Rhein,[32] whereby magnesium can be determined on 0.1 ml. aliquots, with use of a photoelectric colorimeter equipped with a 50 mm. horizontal

cuvette, thereby increasing the light path. A macro modification of the Titan Yellow technique is the control method used for the determination of serum magnesium in this investigation.

MATERIALS AND REAGENTS

1. *Five Milliliter Micro Burette, Graduated in 0.01 or 0.02 ml.*—In the absence of this item, an expedient may be introduced by converting a calibrated 2.0 ml. serologic pipette, graduated in 0.01 ml., to use as a burette. This is a modification of the popular laboratory technique whereby a 2-inch length of rubber tubing containing a tightly-fitted glass bead is used as a stopcock. In this case, the bead-in-tube device is attached to the upper end of the pipette, with the inclosed bead resting on the mouth of the pipette. By a pinching of the bead, air may be admitted or removed to control the emptying or filling of the pipette. The tip end, below the 1.8 ml. mark, is drawn out in a flame to a fine capillary. To be filled, the capillary end is immersed in the solution to be used; gentle suction is applied to the open end of the rubber device during the pinching of the rubber-inclosed bead; when the pipette is full, pressure on the bead is released, and the pipette is fixed in a vertical position. Titration is accomplished by the controlling of the flow of liquid dropwise from the tip by finger pressure on the bead.

2. *Test Tubes.*—Approximately 12 mm. × 100 mm.

3. *Pipettes.*—"Micro Normax, TC," calibrated to contain 0.1 ml. at 20° C.

4. *Disodium Dihydrogen Ethylenediaminetetraacetate.*—225 mg. of SED* is dissolved in freshly distilled water and diluted to 100 ml. Extreme accuracy with this quantity is not required, as this solution, after dilution, is standardized against a calcium standard for every set of titrations. The solution is stable for about one month when stored at 4° C. For use, this stock solution is diluted 1:25 with freshly distilled water.

5. *Stock Calcium Standard.*—Purest reagent grade calcium carbonate (fine chalk powder) is dried in a 110° C. oven overnight and cooled in a desiccator. 2.0018 Gm. is dissolved in a minimum volume of 6 N hydrochloric acid in a small beaker. Care must be taken to prevent loss through effervescence. After heating to dryness on a steam bath, to the absence of fumes of hydrochloric acid, the residue of calcium chloride is quantitatively transferred, by means of washing with small volumes of freshly distilled water, to a 100 ml. volumetric flask and diluted to the mark. A working standard is prepared by dilution of this stock standard 1:100 with freshly distilled water, which gives a concentration of 4.00 meq. of calcium per liter.

6. *Buffer.*—Approximately 0.17 M ethanolamine is prepared by the dilution of 10.2 ml. of purest ethanolamine† to one liter with freshly distilled water. This solution is stored in a tightly sealed glass-stoppered bottle.

7. *Indicator.*—One gram of Eriochrome Black T dye‡ is dissolved in 250 ml. of pure methanol. To this is added 10 ml. of concentrated ammonium hydroxide. This solution is stored at 4° C.

8. *Buffer-Indicator Mixture.*—This must be freshly prepared before use, as it is stable only 2 to 3 hours. Four drops of Eriochrome Black T indicator solution are added to each 10 ml. of ethanolamine solution required. The color of the resulting mixture should be pure blue. If a trace of red or purple is detected, trace amounts of diluted SED solution are added and mixed by swirling, until a pure blue is obtained.

Note: It is important that all apparatus be free of traces of calcium and magnesium, such as are found in tap water, contaminated distilled water, or in glassware which has been contaminated with dust. All vessels (flasks, test tubes, pipettes, etc.) are well washed with a standard type detergent, such as "Alconox," rinsed with hot tap water, and then rinsed several times with freshly distilled water. Vessels are permitted to dry by being inverted on pads of clean filter paper.

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*Bersworth Chemical Company, Framingham, Mass.
†Eastman Kodak "Highest Purity"
‡Hartman-Leddon Co., Philadelphia, Pa.

METHODS AND RESULTS

Method of Investigation and Applicability.—The following is an outline of the approach used in applying this method to hospital patients.

Fresh serum from 107 patients was analyzed for calcium by the proposed method. On the same day, the Clark-Collip procedure was set up in duplicate on the same serum, permitted to stand overnight to insure complete precipitation, and completed the following day.

With a 0.1 ml. "Normax TC" pipette, a sample of the serum was transferred to a test tube containing 2 ml. of the mixed buffer-indicator solution, rinsing the pipette three times with the solution. In a similar manner 0.1 ml. of the 4.00 meq. per liter calcium standard was transferred to a test tube containing 2 ml. of buffer-indicator mixture. The serum was titrated with SED solution, contained in a micro burette, to the blue-green end point of a serum sample plus buffer-indicator solution to which an excess of SED had been added. This control is necessary due to the variation in chromogens of different sera. The calcium standard was then titrated with SED to the blue end point of the fresh buffer-indicator mixture. As equal volumes of standard and serum were used, the total calcium plus magnesium, in milliequivalents per liter, was calculated by the following simple relationship of Sobel:

$$\text{meq./L. (Ca plus Mg)} = \frac{\text{Titration value for serum}}{\text{Titration value for standard}} \times 4.$$

The value "4" represents the concentration of the standard in milliequivalents per liter (meq./L.).

The serum calcium value was estimated by the subtraction of the mean value for serum magnesium in humans (1.64 meq./L., equal to 2 mg. per cent) from this total. Normal serum calcium values were taken as 4.50 to 5.50 meq./L. (9 to 11 mg. per cent). Values in milligrams per cent may be easily obtained by the doubling of the value for calcium in milliequivalents per liter.

Comparison of Results.—Twenty representative values, arranged in order of descending calcium levels, obtained on the 107 specimens are shown in Table I. Both methods were performed in duplicate. Three low values, three elevated, and fourteen normal values are reported. Equally good agreement between the two methods was obtained for those values not reported.

TABLE I. COMPARISON OF SERUM CALCIUM VALUES
(Values in Milliequivalents Per Liter)

COL. 1 TOTAL (CA + MG) IN DUPLICATE			COL. 2 ESTIMATED CA (COL. 1 MEAN MINUS 1.64)	COL. 3 TOTAL CA (CLARK-COLLIP IN DUPLICATE)			COL. 4 DIFFERENCE (COL. 2 MINUS COL. 3 MEAN)
1	2	MEAN		1	2	MEAN	
8.49	8.59	8.54	6.90	6.95	7.05	7.00	−0.10
7.75	7.89	7.82	6.18	6.21	6.01	6.11	0.07
7.64	7.82	7.73	6.09	6.06	6.20	6.13	−0.04
7.17	7.17	7.17	5.53	5.42	5.50	5.46	0.07
6.90	7.00	6.95	5.31	5.08	5.28	5.18	0.13
6.88	7.00	6.94	5.30	4.96	5.12	5.05	0.25
6.83	6.98	6.91	5.27	5.16	5.10	5.13	0.14
6.80	6.98	6.89	5.25	5.10	5.20	5.15	0.10
6.67	6.83	6.75	5.11	5.03	5.29	5.16	−0.05
6.68	6.76	6.72	5.08	5.16	5.10	5.13	−0.05
6.57	6.59	6.58	4.94	4.89	5.11	5.00	−0.06
6.49	6.59	6.54	4.90	4.81	4.85	4.83	0.07
6.51	6.47	6.49	4.85	4.62	4.78	4.70	0.15
6.35	6.45	6.40	4.76	4.84	4.88	4.86	−0.10
6.29	6.51	6.40	4.76	4.61	4.79	4.70	0.06
6.30	6.28	6.29	4.65	4.59	4.71	4.65	0.00
6.10	6.28	6.19	4.55	4.66	4.83	4.75	−0.20
5.83	6.04	5.94	4.30	3.99	4.17	4.08	0.22
5.63	5.67	5.65	4.01	4.08	4.30	4.19	−0.08
4.66	4.82	4.74	3.10	3.13	3.27	3.20	−0.10

Additional Checks on Method.—A further check was made on the validity of the titrametric values for the sum of calcium and magnesium and on the dependability of subtracting the mean magnesium value for human serum.

Random serum samples were analyzed, in duplicate, for calcium by the Clark-Collip method and, likewise, for the sum of calcium and magnesium by the SED titrametric method under evaluation. In addition, the supernatant solution from the oxalate precipitation in the Clark-Collip method was analyzed, in duplicate, for magnesium by a modification of the Titan Yellow technique.

The results, Table II, show the SED titrametric equivalent for the sum of calcium and magnesium compares favorably with the sum of the independently determined calcium concentration (Clark-Collip) and the magnesium concentration (Titan Yellow). It can also be seen that the serum magnesium value of 1.64 meq./L. represents a satisfactory mean.

TABLE II. COMPARISON OF CALCIUM AND MAGNESIUM VALUES
(Concentrations in Milliequivalents Per Liter)

COL. 1 TOTAL (CA + MG)	COL. 2 SERUM CA (CLARK-COLLIP)	COL. 3 SERUM MG* (TITAN YELLOW)	COL. 4 SUM OF CA + MG (COL. 2 + COL. 3)
7.34	5.77	1.60	7.37
7.08	5.55	1.59	7.14
6.95	5.38	1.77	7.15
6.78	5.05	1.60	6.65
6.70	5.05	1.63	6.68
6.68	4.90	1.87	6.77
6.60	4.95	1.87	6.82
6.42	4.75	1.65	6.40
6.34	4.85	1.66	6.51
6.29	4.69	1.69	6.38
6.22	4.70	1.55	6.25
6.19	4.48	1.78	6.26
6.09	4.64	1.60	6.24
5.91	4.18	1.55	5.73
5.71	4.14	1.68	5.82

*The values given represent the mean of two determinations. In no instance did these determinations differ by more than 0.30 meq./L. Mean: 1.67 meq./L.

Method Employed for Serum Magnesium.—The principle on which this control method is based is that of Kolthoff, and the procedure used is a modification of those of Garner, Heagy, Orange and Rhein, and others.

After the centrifuging of the precipitate of calcium oxalate, from the 2 ml. of serum in the method of Clark-Collip, 4.0 ml. of the supernatant (equivalent to 1.6 ml. of serum) was deproteinized with 6.0 ml. of 5 per cent trichloracetic acid. If calcium is not being determined, 2.0 ml. of serum, diluted with 2.0 ml. of water, may be used directly. The mixture was filtered through Whatman No. 42 filter paper and 5.0 ml. of the clear filtrate added to 3.0 ml. of distilled water in a 20 ml. test tube. A reagent blank was prepared from 3.0 ml. of 5 per cent trichloracetic acid and 5.0 ml. of distilled water. Four standards were prepared by the dilution of 0.5, 1.0, 1.5, and 2.0 ml. of magnesium standard (2.00 meq./L.) each with 1.5 ml. 5 per cent trichloracetic acid and by the addition of sufficient water to make the total volume of each standard tube 8.0 ml.

To all tubes were added: 1.0 ml. 0.1 per cent polyvinyl alcohol (aqueous), 1.5 ml. 0.05 per cent Titan Yellow (aqueous), and 2.0 ml. 4 N sodium hydroxide. All tubes were well mixed by inversion, and the colors, which are stable for three hours, were read against a water blank in a Coleman Junior spectrophotometer (Model 6) at 560 mμ. True densities of standards and unknowns were obtained by subtraction of that of the reagent blank. A graph of the standard values was plotted "optical density" vs. "magnesium concentration in meq./L." Values for the unknowns were read from this graph in the usual manner, with due care given to appropriate correction factors for dilution.

Reproducibility of Proposed Technique.—For the determination of the reproducibility of the proposed procedure for serum calcium estimation, multiple determinations were performed on several serum samples. The results of two such analyses are shown in Table III.

TABLE III. REPRODUCIBILITY OF PROPOSED PROCEDURE
(Calcium Concentrations in meq./L.: Serum 1 4.97; Serum 2 3.37)

CLARK-COLLIP PROCEDURE			
SERUM 1		SERUM 2	
DETERMINATION NUMBER	ESTIMATED CONCENTRATION	DETERMINATION NUMBER	ESTIMATED CONCENTRATION
1	4.96	1	3.55
2	4.80	2	3.47
3	4.88	3	3.41
4	4.88	4	3.46
5	4.92	5	3.50
6	4.88	6	3.49
7	4.92	7	3.44
8	4.92	8	3.43
9	4.84	9	3.41
10	4.80	10	3.46
Average concentration:	4.88	11	3.49
Standard deviation:	±0.053	12	3.49
		Average concentration:	3.46
		Standard deviation:	±0.041

Recovery of Added Calcium.—Analyses were made to determine whether known quantities of calcium, which had been added to pooled serum, could be recovered. Results of two of the recovery experiments are indicated in Table IV.

TABLE IV. RECOVERY OF ADDED CALCIUM

	CALCIUM ADDED (MEQ.)	CALCIUM RECOVERED (MEQ.)	PER CENT RECOVERY
Serum A	1.00	1.04	104
		1.02	102
	2.00	1.91	96
		1.93	97
	3.00	2.97	99
		3.06	98
	4.00	4.11	103
		4.15	96
Serum B	1.00	0.96	96
		1.02	102
	2.00	1.95	98
		2.05	103
	3.00	3.08	103
		3.01	100
	4.00	4.16	104
		4.12	103

Effect of Hemolysis.—As the proposed method initially determines the sum of calcium and magnesium, and as there is a greater concentration of magnesium in the cells than in the serum, the danger of false high values through use of hemolyzed serum was investigated.

A series of freshly-drawn blood specimens were permitted to clot and then centrifuged. A portion of the clear unhemolyzed serum of each was drawn off for analysis. By means of a glass rod each original specimen, containing clot and serum, was vigorously stirred to cause hemolysis. The tubes were then centrifuged. Both the hemolyzed and unhemolyzed serum from each specimen was analyzed by the proposed method. As seen in Table V, no significant increases in titration values were observed.

TABLE V. EFFECT OF HEMOLYSIS

TITRAMETRIC EQUIVALENT OF TOTAL (CA + MG) IN MEQ./L.						
UNHEMOLYZED			HEMOLYZED			
(A)	(B)	MEAN	(A)	(B)	MEAN	DEVIATION
6.98	6.90	6.94	6.99	7.11	7.05	0.11
6.87	6.93	6.90	6.70	6.92	6.94	0.04
6.97	6.79	6.88	6.79	6.96	6.87	−0.01
6.83	6.80	6.82	6.83	6.95	6.89	0.07
6.71	6.89	6.80	6.70	6.86	6.78	−0.02
6.48	6.59	6.53	6.50	6.61	6.56	0.03
6.42	6.58	6.50	6.66	6.55	6.61	0.11
6.26	6.44	6.35	6.40	6.39	6.40	0.05
6.24	6.40	6.32	6.50	6.30	6.40	0.08
5.63	5.75	5.69	5.61	5.68	5.64	−0.05

TABLE VI. EFFECT OF DEGREE OF HEMOLYSIS

HEMOGLOBIN CONTENT (GRAMS %)	TOTAL (CA + MG) MEQ./L.			DEVIATION FROM CONTROL
	A	B	MEAN	
0.00	6.36	6.28	6.32	Control
0.02	6.30	6.27	6.29	−0.03
0.04	6.25	6.34	6.30	−0.02
0.06	6.44	6.28	6.34	0.02
0.08	6.33	6.26	6.30	−0.02
0.10	6.36	6.37	6.36	0.04
0.12	6.44	6.34	6.39	0.07
0.15	6.44	6.32	6.38	0.06

TABLE VII. EFFECT OF SERUM TOTAL PROTEIN CONCENTRATION
(Each Value Reported Represents the Mean of Duplicate Determinations)

TOTAL PROTEIN (GRAMS %) (BIURET METHOD)	TOTAL CALCIUM (CLARK-COLLIP) (MEQ./L.)	ESTIMATED TOTAL CALCIUM (THIS METHOD) (MEQ./L.)
4.8	4.10	4.20
5.5	5.08	5.01
6.8	4.72	4.75
7.0	4.95	4.90
7.5	5.13	5.15
6.2	4.93	4.85
6.5	4.62	4.55
6.7	4.60	4.50
6.7	4.70	4.77
7.7	4.80	4.71
8.1	5.42	5.55
8.9	5.75	5.69
9.3	5.91	5.95
10.0	5.11	5.03
10.1	5.83	5.99

A further investigation was conducted to determine the degree of hemolysis necessary to cause increased titration values. A series of serum specimens was prepared from pooled serum in increasing degrees of hemolysis, the degree being based on controlled hemoglobin concentrations. Known volumes of clear, unhemolyzed pooled serum were pipetted into each of eight test tubes; to each was added calculated, and increasing, volumes of a prepared hemolysate; each tube was then made up to a constant volume with normal saline solution (0.85 per cent sodium chloride in distilled water). The hemolysate was prepared by hemolyzing washed group "O" red cells in a minimum quantity of distilled water and by filtering the resulting solution free of solid particles. The range of serum hemoglobin concen-

trations thus prepared was determined to be from 0.00 per cent to 0.15 per cent. Duplicate SED titrations were made on each serum specimen to determine the sum of calcium and magnesium. Results are shown in Table VI.

It will be noted that blood hemolyzed to a degree wherein the serum contains 0.15 per cent hemoglobin is well beyond that encountered in routine clinical laboratory work.

Effect of Total Serum Protein Concentration.—Sera of low, normal, and slightly elevated total protein concentrations were analyzed for calcium by the control method of Clark-Collip, in conjunction with an estimation of serum calcium by the proposed method. Results of analyses are shown in Table VII.

The close agreement of serum calcium values by the two methods was deemed sufficient evidence that there is no appreciable time lag for the formation of the red soluble calcium dye complex, either by free or protein-bound calcium in serum. Chelation of the dye-bound calcium is also immediate. However, it was observed that in the course of the titration with SED, as the blue-green end point is reached, an interval of 30 to 40 seconds between drops is necessary to permit the color to stabilize. The upper extent of the time limit stated applies particularly in serum with high protein concentration.

DISCUSSION

The determination can be made on 0.1 ml. of serum, which is a more practical compromise for clinical use between the ultramicro volume of 0.02 ml., and the macro quantity of 2.0 ml. Time for a single determination need not exceed twenty-five minutes. The results of such analysis can enable the clinician to begin early treatment of a convulsant patient with the benefit of prompt objective evidence.

Of the 107 serum samples, on which determinations were made by the proposed method, and that of Clark and Collip, the maximum difference between the two values was 0.40 meq. per liter, the average being 0.11 meq. per liter. The accuracy of the titration for the determination of total calcium plus magnesium, from which a mean value of 1.64 meq. per liter for magnesium is subtracted, was substantiated. This total agreed with the sum of the results of separate analysis for calcium, the method of Clark and Collip being used, and for magnesium on the same serum, by a modification of the Titan Yellow method. The effects of slight hemolysis and of high total protein content on the determination of calcium in serum by the proposed method were studied and found to be negligible.

A chief weakness of the method is the assumption of the value 1.64 meq./L. for magnesium. Although it has been shown that serum magnesium values rarely deviate unduly from this mean, a case has been reported wherein convulsions were directly traced to low serum magnesium levels.[33] Therapeutically, magnesium may produce high serum magnesium levels, thus giving false high calcium values by this method.

SUMMARY

1. A simple titrametric method has been presented for the rapid estimation of calcium in 0.1 ml. of serum.

2. The use of chelation powers of disodium ethylenediaminetetraacetate and the role of azo dyes as indicators in the determination of calcium and magnesium has been reviewed. It is upon these principles that the proposed method is based.

3. Data are given to show the satisfactory agreement between the proposed method and that of Clark and Collip.

4. A modified colorimetric macromethod for the determination of magnesium in serum and urine, and devised for use as a control procedure in this investigation, is presented.

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IMPROVED MICROMANOMETRIC METHODS FOR THE ANALYSIS OF RESPIRATORY GASES IN PLASMA AND WHOLE BLOOD

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NATELSON[1] described a micromanometric method for the estimation of CO_2 content of plasma based on the principles of the Van Slyke-Neill[2] method. The method of Natelson requires 0.03 ml. of plasma for each determination, and determinations are carried out in the Kopp-Natelson microgasometer. There was a mean deviation of −1.3 vol. per cent from the expected value when the method was tested with standard Na_2CO_3 solutions, and there was a standard deviation of individual determinations about the mean of 1.3 vol. per cent. The present work was undertaken to improve the accuracy and sensitivity of the method and to extend it to the combined determination of oxygen and carbon dioxide in whole blood.

METHODS

In the main, the information concerning manipulation and maintenance of the apparatus which is included in the instruction book accompanying the Kopp-Natelson microgasometer Model 600 is appropriate to the following methods. Since the accuracy and sensitivity of these methods depend on the preparation and use of reagents and upon modifications in the method of calculation, these procedures will be described in detail.

The concentrations of the reagents are approximately the same as those employed in the macro methods; the volumes are reduced appropriate to the dimensions of the microgasometer. All reagents are made up with acidulated, boiled distilled water, are degassed, and are stored in mercury-sealed syringes, which permit convenient delivery into the microgasometer (see Fig. 1). Degassing may be accomplished in a Van Slyke manometric apparatus.

Collection syringes for blood samples are prepared in the following manner: The plunger of a 2 mm. syringe is coated with light stopcock grease; the dead space in the hub of the syringe is filled with a solution consisting of one part of saturated sodium fluoride solution and 10 parts of heparin solution, 50,000 units per milliliter. Immediately after a blood sample has been obtained, a droplet of mercury is introduced into the syringe through a fine-bore needle. Air bubbles are then expelled from the syringe, the hub is sealed with a segment of waxed toothpick, and the syringe is agitated gently but thoroughly to prevent clotting. When the sample is to be used for analysis of plasma, the collection syringe is prepared in the fashion described by Davenport[3] and is centrifuged immediately. The plasma is then transferred anaerobically from the syringe through a U-shaped hypodermic needle into a 7 mm. inner diameter test tube containing mercury and a 2 cm. column of mineral oil. When the sample is to be used for analysis of whole blood it is stored in the refrigerator until analysis. The blood is kept mixed by slow rotation of the sealed collection syringe on an electric mixing device, which revolves 15 to 20 times per minute (Fig. 2). The mixer may be plugged into the light socket within the refrigerator if the door switch is shunted.

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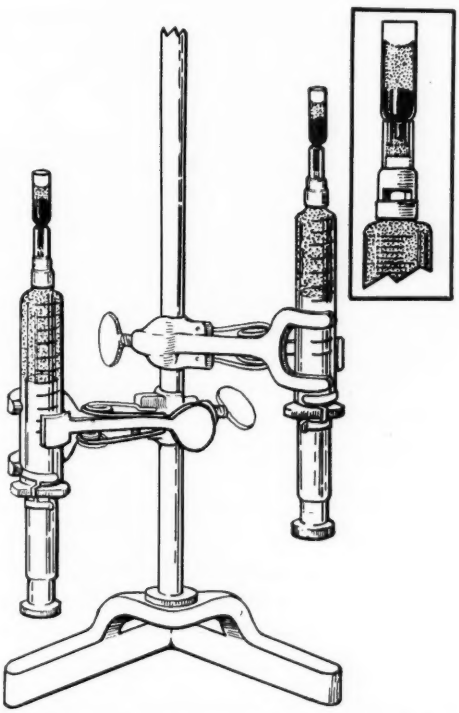

Fig. 1.—A convenient method for storage and delivery of degassed reagents into the microgasometer. The reagent is transferred to a greased 10 ml. syringe after being degassed. A glass cup, 5 mm. × 20 mm., terminating in a 1 mm. capillary, is fitted to the hub of the syringe with tygon tubing. Mercury is placed in the cup to act as a vapor seal. Reagent is forced through the mercury for delivery into the microgasometer.

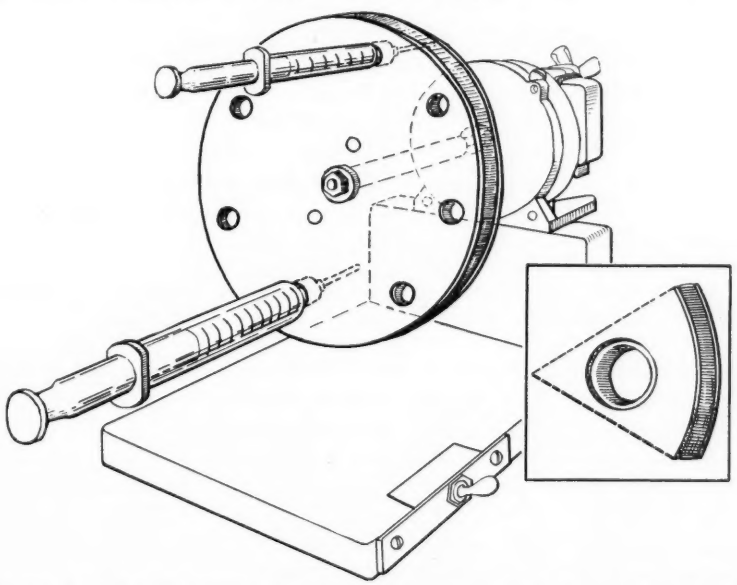

Fig. 2.—A simple device for preventing sedimentation of whole blood stored in luer-lok syringes of any size. The shaft of a 15 r.p.m. electric motor (Merkle-Korff Flexo-Action Motor) is extended 2½ inches to support the plate. The plate is constructed of two sheets of 5/64" aluminum, bolted on either side of a sheet of 1/4" rubber. Holes 13/32 inches in diameter (1/64 inch larger than the luer-lok syringe hub) are drilled in the aluminum; holes 5/16 inches in diameter are drilled in the rubber as shown in the insert. A motor switch is mounted in the wooden base.

The Determination of Plasma CO_2 Content.—

Reagents:

1 N Lactic acid
Caprylic alcohol
1 N NaOH
Water (degassed)

Procedure: Plasma, in the amount of 0.03 ml., is measured into the pipette of the microgasometer from a test tube containing mercury and a 2 cm. column of mineral oil. Before the drawing in of the sample, a droplet of mercury is allowed to fall from the tip of the pipette, to avoid aspiration of air with the sample. This maneuver is repeated with each reagent. The sample is followed by 0.01 ml. seal of mercury obtained by immersing the pipette into the mercury at the bottom of the test tube. A 2 to 3 mm. column of caprylic alcohol is placed in the delivery cup of the mercury-sealed syringe containing degassed lactic acid. Lactic acid is forced through the mercury into the delivery cup. Lactic acid, in the amount of 0.01 ml., is taken into the pipette, followed by approximately 0.002 ml. of caprylic alcohol and a mercury seal. One-tenth milliliter of degassed water is taken up, and mercury is drawn into the pipette to the 0.12 ml. mark in the reaction chamber. The chamber stopcock is closed and the chamber is evacuated to just below the lower shoulder and shaken for two minutes. The liquid level is returned smoothly to the 0.12 ml. mark and P_1 is read. The pressure in the chamber is returned to atmospheric presure by the allowing of the mercury to just fill the manometer. The stopcock is opened cautiously. The delivery cup of the sodium hydroxide syringe is filled and 0.02 ml. of NaOH is drawn into the pipette, followed by a mercury seal. Mercury is drawn into the 0.12 ml. mark of the chamber, and the chamber stopcock is closed. The chamber is evacuated as before and shaken for ten seconds. The liquid meniscus is returned to the 0.12 mark and P_2 is read. The temperature in the region of the chamber is noted.

The contents of the chamber are then discarded and the chamber is washed successively with 0.1 ml. portions of Haemo-sol solution, water, 1 normal lactic acid, and water. These solutions need not be degassed, however, washing is performed in a closed, evacuated chamber to prevent inclusion of air in the mercury or in the detritus, which accumulates in the trap below the chamber.

Calculations: A correction factor (C) is obtained by the performing of blank determinations in which degassed distilled water is substituted for plasma. The correction factor is the mean of the differences between P_1 and P_2. The following formula is used to calculate CO_2 content:

$$(CO_2)_p = (P_1 - P_2 - C)\ F.$$

The factors (F) for various temperatures are calculated from the formula of Van Slyke,[4] on the basis of the volumes of reagents and sample given above, a total chamber volume of 2.9 ml., and a reabsorption factor for CO_2 (i) of 1.017. These are listed in Table I. A chamber volume of 2.9 ml. is used, because the 3.0 ml. calibration is placed low in the narrow stem below the expanded portion of the chamber, and the liquids cannot be shaken adequately when the mercury is lowered to the 3.0 ml. mark.

TABLE I. CONVERSION FACTORS FOR PLASMA CO_2 CONTENT

T. °C.	F (VOL. %)	F (mM/L.)	T. °C.	F (VOL. %)	F (mM/L.)
20	0.5208	0.2339	25.5	0.5078	0.2281
20.5	0.5197	0.2334	26	0.5066	0.2276
21	0.5185	0.2329	26.5	0.5055	0.2271
21.5	0.5173	0.2324	27	0.5044	0.2266
22	0.5160	0.2318	27.5	0.5033	0.2261
22.5	0.5148	0.2313	28	0.5022	0.2256
23	0.5136	0.2307	28.5	0.5011	0.2251
23.5	0.5124	0.2302	29	0.5001	0.2246
24	0.5112	0.2296	29.5	0.4991	0.2241
24.5	0.5101	0.2291	30	0.4980	0.2237
25	0.5089	0.2286	30.5	0.4970	0.2232

The Combined Determination of Oxygen and Carbon Dioxide Contents of Whole Blood.—Reagents:

1 N Lactic acid
Caprylic alcohol
Water (degassed)
3.2 per cent Potassium ferricyanide containing 0.8 per cent saponin
1 N NaOH
25 per cent Sodium hydrosulfite in 1 normal NaOH

It was found that potassium ferricyanide, when allowed to stand in contact with mercury for more than a few minutes or when stored in the refrigerator for more than 48 hours, was responsible for an increase in the CO_2 correction factor (C). Therefore the potassium ferricyanide is prepared fresh each morning, is degassed and stored in a lightly greased syringe sealed with a waxed toothpick. Immediately before use, a 1 cm. column of potassium ferricyanide is carefully layered over mercury in a 7 mm. inner diameter test tube, from which the reagent is drawn into microgasometer pipette. All other reagents are degassed and stored in mercury-sealed dispensing syringes (Fig. 1). Thoroughly mixed blood is transferred immediately before analysis from the collected syringe to a 5 mm. inner diameter test tube containing mercury and a 1 cm. column of mineral oil. It was found that an average of 20 seconds elapsed between the time the mixed whole blood was discharged from the syringe and the time it was drawn into the microgasometer pipette. It was felt that an insignificant degree of sedimentation occurred during this interval.

Procedure: Blood, in the amount of 0.03 ml., is drawn into the pipette and followed by a mercury seal; 0.01 ml. of lactic acid and approximately 0.003 ml. of caprylic alcohol are measured into the pipette, followed by a mercury seal. Degassed water, 0.06 ml. in amount, is measured into the pipette as two consecutively drawn 0.03 ml. portions separated by a short column of mercury. This is followed by a mercury seal. Three-hundredths milliliter of ferricyanide-saponin solution is measured into the pipette. Mercury is then taken into the 0.12 ml. mark of the chamber. The chamber stopcock is closed and the mercury level is depressed to the lower shoulder of the chamber. The chamber is shaken for three minutes. The fluid level is returned slowly and evenly to the 0.12 ml. mark and the pressure (P_1) is read. The chamber is returned to atmospheric pressure and the stopcock is opened; 0.02 ml. of NaOH is drawn into the pipette, followed by mercury to the 0.12 mark. The chamber is closed, evacuated, and shaken for 10 seconds. The pressure (P_2) is read with the meniscus at the 0.12 mark. The chamber is returned to atmospheric pressure and opened; 0.02 ml. of sodium hydrosulfite solution is taken in and followed by a mercury seal to the 0.12 mark. The chamber is closed and evacuated as before. The chamber is shaken for 20 seconds, following which the liquid level is returned to the 0.12 ml. mark and the pressure (P_3) is read.

The contents of the chamber are discarded and the chamber is washed successively with sodium hydroxide, Haemo-sol solution, water, lactic acid, and water.

Occasionally, during analysis of whole blood, mercury and solution tend to remain in the constricted portion of the chamber beneath the stopcock when the chamber is evacuated. Painting a small area immediately beneath the stopcock with picolite, a silicone, or some other hydrophobic material reduces this tendency. Following closure of the chamber stopcock, the chamber should be evacuated slowly. If some solution threatens to stay in the constricted portion, the chamber pressure should be returned to atmospheric immediately, to allow the small bubble of extracted gas which will have formed to rise above the liquid.

Calculations: Correction factors (C) for calculation of CO_2 and O_2, determined as the mean differences of $P_1 - P_2$ and $P_2 - P_3$, respectively, are obtained from blank analyses

in which degassed water is substituted for whole blood. Two additional corrections are required for calculation of the carbon dioxide and oxygen contents of whole blood. It was observed that smaller amounts of carbon dioxide were recovered from whole blood by the micromanometric method than by the Goldstein method, when calculated with the factors used for the plasma determination. This discrepancy was not observed when water solutions of sodium carbonate were analyzed with use of the whole blood reagents. It was found that the discrepancy was proportional to the amount of either whole blood or plasma protein present in the sample being analyzed (see Fig. 3). Solutions of bovine albumin and fibrinogen with added $NaHCO_3$ exhibited differences similar to blood and plasma. The error was not related to viscosity, since solutions of dextran, a high molecular weight carbohydrate, gave the same recoveries as water solutions of bicarbonate. It was also noted that the discrepancy was proportional to the CO_2 content. Analyses of 22 blood samples from different subjects revealed that the mean recovery of CO_2 by the micromanometric method was 4.3 per cent less than that obtained with the Goldstein method; the standard error of the mean difference was 0.27 per cent. In accordance with this information, the correction factor, "*i*," which in the Van Slyke formula accounts for the amount of CO_2 which is reabsorbed during return to the reading volume and which has been found to be 1.017 in the plasma determination, was increased to 1.060 to obtain the factors presented in Table II for calculation of whole blood CO_2 content.

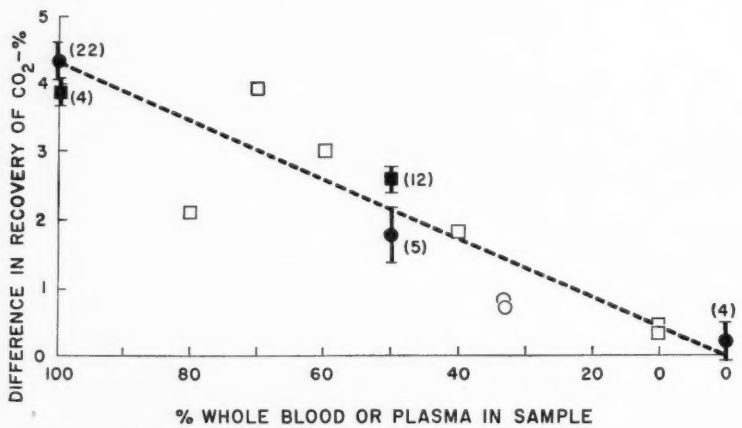

Fig. 3.—Effect of blood proteins on recovery of CO_2 by the micromanometric method for whole blood. The ordinates represent the amount of CO_2 which the micromanometric method failed to detect, expressed as percentage of the total CO_2 content determined by the method of Goldstein. The conversion factors for the micro method were based on a CO_2 reabsorption factor, "*i*," of 1.017. The abscissae indicate the dilution of the samples with water or Na_2CO_3 solution expressed as percentage of whole blood or plasma in the samples. The solid circles and squares represent average values for whole blood and plasma, respectively; the numbers in parentheses indicate the number of samples contributing to each average; the vertical lines are one standard error of the mean. The open circles and squares represent single samples. The dashed line was drawn arbitrarily from the mean value obtained from whole blood samples to zero for samples containing no protein.

The second correction is derived from the observation that calculation of the oxygen content from the formula of Van Slyke yielded results consistently 0.6 vol. per cent lower than those obtained with the macro method. Investigation of this discrepancy indicated that there was a consistent disappearance of oxygen from the sample independent of the concentration of oxygen in the sample and independent of the nature of the sample (Table III). The same amount of oxygen disappeared from water samples as from whole blood samples. This discrepancy is corrected by adding 0.60 vol. per cent to the calculated O_2 content.

TABLE II. CONVERSION FACTORS FOR WHOLE BLOOD CO_2 AND O_2 CONTENT

	F CO_2		F O_2		F CO_2		F O_2
T. °C.	(VOL. %)	(mM/L.)	(VOL. %)	T. °C.	(VOL. %)	(mM/L.)	(VOL. %)
20	0.5411	0.2431	0.4896	25.5	0.5276	0.2370	0.4802
20.5	0.5399	0.2425	0.4888	26	0.5267	0.2366	0.4793
21	0.5388	0.2421	0.4879	26.5	0.5255	0.2361	0.4785
21.5	0.5375	0.2415	0.4870	27	0.5243	0.2355	0.4776
22	0.5363	0.2409	0.4861	27.5	0.5230	0.2350	0.4768
22.5	0.5350	0.2403	0.4853	28	0.5221	0.2345	0.4759
23	0.5338	0.2398	0.4844	28.5	0.5210	0.2341	0.4751
23.5	0.5326	0.2393	0.4836	29	0.5200	0.2336	0.4743
24	0.5314	0.2387	0.4827	29.5	0.5189	0.2331	0.4735
24.5	0.5301	0.2381	0.4819	30	0.5178	0.2326	0.4727
25	0.5290	0.2376	0.4810	30.5	0.5167	0.2321	0.4719

TABLE III. RECOVERY OF OXYGEN FROM BLOOD AND FROM WATER

O_2 IN BLOOD (VOL. %)			O_2 IN WATER (VOL. %)		
GOLDSTEIN	MICRO	DIFF.	GOLDSTEIN	MICRO	DIFF.
4.59	4.07	−0.52	2.1	1.5	−0.6
4.92	4.87	−0.05	2.1	1.4	−0.7
9.42	8.98	−0.44	4.4	3.5	−0.9
10.57	10.29	−0.28	4.4	3.6	−0.8
11.00	10.12	−0.88	4.9	3.5	−1.4
11.33	10.90	−0.43	6.3	5.9	−0.4
12.04	11.51	−0.53	6.9	6.3	−0.6
12.90	12.20	−0.70	7.2	6.5	−0.7
13.19	12.53	−0.66	7.3	6.5	−0.8
13.95	13.23	−0.72	8.1	6.7	−1.4
15.53	15.33	−0.20		m	= −0.83
15.91	14.96	−0.95		s.d.	= ±0.33
18.03	16.63	−1.40		s.e.	= ±0.10
19.72	18.37	−1.35			
21.22	20.07	−1.15			
21.32	21.29	−0.03			
		m = −0.643			
		s.d. = ±0.42			
		s.e. = ±0.10			

Each figure represents the average of duplicate determinations. The blood determinations are arranged in ascending order of oxygen content to emphasize the fact that the discrepancies are independent of oxygen content. The range of oxygen contents in water were obtained for the micro analyses by substitution of various volumes of water saturated with 100 per cent oxygen at room temperature for part of the degassed water used in the micro method. The comparable values listed for the macro method were obtained by multiplying the recovery obtained from a 1.0 ml. sample by a factor chosen to correspond with the volume of 0_2 saturated water analyzed by the micro method.

The formula for calculation of the CO_2 content of whole blood is:

$$CO_2 = (P_1 - P_2 - C_{co_2})\ F_{co_2}.$$

The formula for calculation of the O_2 content of whole blood is:

$$O_2 \text{ (vol. per cent)} = (P_2 - P_3 - C_{o_2})\ F_{o_2} + 0.6.$$

The conversion factors (F) for the whole blood determinations are listed in Table II.

Oxygen Capacity.—Determination of the oxygen capacity of whole blood samples of 1 ml. volume or less may be obtained conveniently in the following manner. After blood has been removed from the refrigerated sample syringe for the second analysis of content, the sample syringe is filled with a volume of room air equivalent to approximately 10 times the volume of blood remaining in the syringe. The syringe is rotated slowly at room temperature for 15 minutes, the air in the syringe being expelled and replaced twice at 5-minute intervals. The blood is then analyzed by the combined method for whole blood described above, with the exception that the pressure obtained after absorption of CO_2 is recorded as P_1 and the pressure after absorption of O_2 is taken to be P_2.

Oxygen capacity is calculated as follows:

$$O_2 \text{ capacity (vol. per cent)} = (P_1 - P_2 - C)\ F_{o_2} + 0.3.$$

The correction, 0.3 volumes per cent, was obtained from comparative analyses of the O_2 capacity of 10 blood samples by the method of Roughton, Darling, and Root[5] and the micromanometric method. This correction factor takes into account the reduced recovery of oxygen by the micro method discussed above and the additional oxygen dissolved in blood at an oxygen tension of 154 mm. Hg at temperatures between 24° and 29° C.[6]

EXPERIMENTAL

The validity of the micro methods was established by comparison with the method of Goldstein and his associates,[7] which has been found[8] to be an acceptable substitute for the Van Slyke-Neill method.[2] Tables IV and V indicate the agreement to be expected from analysis of the same sample of plasma or whole blood by these two methods. No statistically significant differences were detected in any of the comparisons based on the mean of the differences between analyses performed by the macro and micro methods.

The relative sensitivities of the macro and micro methods are compared in Table VI. The reproducibility obtainable with the micro method is partially a function of the skill of the operator. The comparisons of operator "B" in Table VI suggest that the micro methods are capable of the same sensitivity as the macro methods, although operator "A" obtained sensitivities with the micro method for analysis of whole blood which were one-half of those she obtained with the micro method.

The sensitivity of the micro methods can probably be increased by encasing the reaction chamber in a water-filled glass jacket and by providing for continuous shaking with an electric motor. These refinements were tested and found to be convenient. It has not been determined whether or not they are essential for optimal performance. A microgasometer equipped with water jacket, electric motor shaker, and illumination of the manometer is illustrated in Fig. 4.

TABLE IV. CO_2 CONTENT OF PLASMA

MACRO (VOL. %)	MICRO (VOL. %)	DIFF. (VOL. %)	
62.3	63.4	+1.1	
62.0	61.8	−0.2	
64.1	63.8	−0.3	
61.9	62.4	+0.15	
63.2	63.4	+0.2	
63.7	63.7	0.0	n = 20
62.9	53.2	+0.3	mean diff = +0.01
52.5	52.7	+0.2	s.d. = ±0.44
51.6	51.2	−0.4	s.e. = ±0.10
51.8	51.6	−0.2	
55.7	55.6	−0.1	
53.3	52.8	−0.5	
43.2	42.8	−0.4	
42.2	42.2	0.0	
65.0	64.8	−0.2	
63.2	64.0	+0.8	
48.0	47.5	−0.5	
48.6	48.1	−0.5	
52.9	53.2	+0.3	
42.5	42.6	+0.1	

Plasma samples analyzed by the Goldstein method and the micromanometric method. Application of the "t" test indicates that no significant difference ($P \gg 0.50$) exists between the results obtained by the two methods.

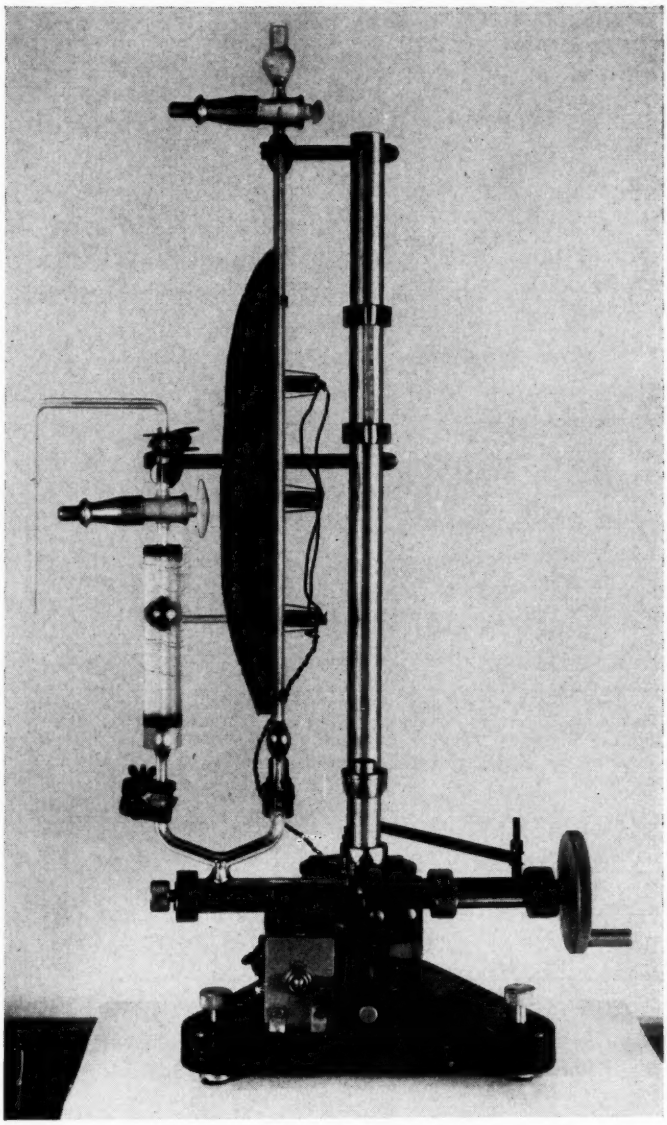

Fig. 4.—The microgasometer has been modified for automatic shaking and illumination of the manometer. A water jacket has been fitted around the chamber for improved temperature control. A 200 r.p.m. electric motor (Merkle-Korff Flexo-Action model SG-15) is mounted on the rear of the base with the shaft parallel to the main support column. The motor is linked to the bar through an adjustable eccentric and connecting rod, which are visible above the piston assembly. A cam attached to the shaker screw activates a microswitch controlling the motor when the shaker screw is released. Illumination of the manometer is provided by three panel lamps mounted in a crescent-shaped bar of Plexiglas, which is supported from the manometer by fuse clips. A horizontal curved rod of Plexiglas mounted opposite the lowest lamp illuminates the 0.12 mark of the chamber. Current for the lamps is supplied from a 6.3 volt filament transformer, which is mounted in the box on the left front section of the base. The box also contains a light switch and the motor control microswitch.

TABLE V

CO_2 VOL. %					O_2 VOL. %				
MACRO		MICRO		DIFF.	MACRO		MICRO		DIFF.
54.76 54.78	54.77	54.79 55.00	54.90	+0.13	10.63 10.65	10.64	10.70 10.60	10.65	+0.01
48.67 48.81	48.74	48.67 48.82	48.75	+0.01	13.01 12.95	12.98	13.25 13.20	13.28	+0.30
59.86 59.55	59.71	59.58 59.42	59.50	−0.21	6.77 6.74	6.76	6.77 6.82	6.80	+0.04
43.30 43.43	43.37	43.61 43.66	43.64	+0.27	9.55 9.58	9.57	9.71 9.61	9.66	+0.09
51.09 51.19	51.14	51.41 51.25	51.33	+0.19	7.25 7.27	7.26	7.25 7.20	7.23	−0.03
53.64 53.65	53.65	53.55 53.76	53.66	+0.01	2.98 3.00	2.99	2.95 2.76	2.86	−0.13
28.02 28.20	28.11	28.34 28.18	28.26	+0.15	13.93 13.76	13.85	13.54 13.64	13.59	−0.26
51.43 51.50	51.47	51.43 51.59	51.51	+0.04	18.09 18.21	18.15	18.27 18.22	18.25	+0.10
56.96 56.76	56.86	56.62 56.51	56.57	−0.29	13.07 13.14	13.11	13.11 12.97	13.04	−0.07
59.00 59.11	59.06	58.93 59.09	59.01	−0.05	6.10 6.15	6.13	6.09 6.14	6.12	−0.01
			m = +0.025					m = +0.004	
			s.d. = ±0.175					s.d. = ±0.149	
			s.e. = ±0.055					s.e. = ±0.047	

Consecutive analyses of whole blood samples in duplicate by the Goldstein method and the micromanometric method. These comparisons were not included in the determination of the corrections for the micromanometric method and were performed by another person.

TABLE VI. VARIANCES WITHIN METHODS

	PLASMA CO_2 (VOL. %)			WHOLE BLOOD CO_2 (VOL. %)		O_2 VOL. %	
"A"	Macro	(108)	0.20	(23)	0.18	(23)	0.10
	Micro	(65)	0.24	(23)	0.28	(23)	0.20
"B"	Macro			(10)	0.11	(10)	0.05
	Micro	(14)	0.09	(37)	0.09	(52)	0.08

The reproducibility of the methods in the hands of two operators, "A" and "B," as measured by the standard deviation of the differences between duplicate determinations (s.d. = $\sqrt{\frac{\Sigma\ (\text{diffs}^2)}{2n}}$ where "n," the numbers in parentheses, is the number of samples analyzed in duplicate by each method).

DISCUSSION

The development of the micromanometric methods described herein was undertaken because of need for a precise but rapid method for detecting sudden alterations in acid-base balance. This aim was realized to the extent that single determinations of plasma CO_2 content can be performed in seven minutes, and whole blood CO_2 and O_2 content can be determined in fifteen minutes with a precision which affects the estimation of CO_2 tension by not more than 0.5 mm. Hg and which can be responsible for an error not exceeding 2 per cent in the estimation of oxygen saturation. Duplicate analyses can be performed easily

with 1 ml. of blood. Natelson's suggestions[1, 9] for obtaining blood samples from infants may be useful for doing analyses on smaller volumes of drawn blood.

One or two weeks of practice with the whole blood method is sufficient training for one who is proficient with the Van Slyke-Neill method. The plasma method may be mastered in a few days.

The sensitivity of a manometric method is a function of the skill and care of the individual performing the analysis. The best performance of a method is limited, however, by the apparatus and chemistry involved. One of the factors which tends to increase the variance of the micromanometric methods as compared with the macromanometric methods is that the ratio of sample volume to reading volume is 0.03/0.12, or 0.25, whereas in the macro method utilizing 1 ml. of sample, the ratio is 0.50. Thus an error in reading the manometer or in adjusting the level of the meniscus at the reading line has twice the effect on the calculated content of gas in the micro methods as it does in the macro methods. A second factor, which may be remediable, is the accuracy with which the manometer of the microgasometer may be read. In its present form the manometer is etched with thick, well-annealed lines extending partially across the front face and has an opal glass stripe on the back surface. There is, furthermore, no provision for illumination of the manometer. These circumstances make it difficult to estimate the level of the mercury meniscus to less than 0.2 mm.

So long as the chemistry of a method is incompletely understood there remains the possibility for indeterminate errors arising from inadequate control of reactions. It is hoped that the causes for the reduced extraction into the gas phase of CO_2 and O_2 in the micro method, as compared with the macro method, will be determined.

SUMMARY

Improved micromanometric methods for analysis of CO_2 content of plasma and CO_2 and O_2 contents of whole blood have been described. Corrections required for analysis of whole blood which are peculiar to the Kopp-Natelson microgasometer have been discussed and incorporated into a table of conversion factors.

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A MICROASSAY FOR PLASMA FIBRINOGEN

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THE methods of determining fibrinogen have been reviewed by Morrison.[1] Fibrinogen can be separated from the plasma by salting it out or by converting it to fibrin by treatment with thrombin. The latter method is more specific and less subject to error when properly done. Early workers supplied the thrombin simply by recalcifying the plasma. More recently, purified thrombin has been added to assure complete clotting of the fibrinogen, and the resulting fibrin has been determined colorimetrically[2] or gravimetrically.[3] However, these methods require 0.5 ml. of plasma for precise results. This limits their usefulness with infants and in small animal research.

In the present method, previous procedures[1-3] have been modified by incorporating ethanol into the coagulation mixture and adopting more sensitive colorimetric and gravimetric methods. The new procedure requires much less thrombin than previous methods and its requires only 0.1 ml. of plasma for reproducible values. Two means of determining the amount of coagulated fibrin are presented. In one the clot is dried and weighed to the nearest microgram; in the other it is nesslerized and assayed colorimetrically.

METHODS

Apparatus: A microbalance is required for the gravimetric procedure.

Reagents:

1. Ten per cent alcoholic saline. Mix 100 ml. of 95 per cent ethanol with 900 ml. of 0.85 per cent saline solution.
2. Thrombin solution. Dissolve about 10 mg. of Topical Thrombin* in 100 ml. of the 10 per cent alcoholic saline solution. This solution contains about 2.5 units of thrombin per milliliter.
3. Reagent grade sulfuric acid.
4. Thirty per cent hydrogen peroxide.
5. Ammonium sulfate standard solution. Dissolve 0.472 Gm. of ammonium sulfate in 1 liter of water. This solution contains 0.10 mg. of nitrogen per milliliter.
6. Nessler's reagent. This is prepared according to the directions of Koch and McMeekin.[4]

Procedure: Citrated or oxalated blood is centrifuged 10 minutes at 2,000 gravity (3,200 r.p.m. in a No. 1 International centrifuge) and 0.1 ml. of the plasma is pipetted into 15 × 85 mm. tubes. About 2 ml. of thrombin solution is added to each tube and quickly mixed with the plasma. Coagulation of the fibrinogen is complete in 2 or 3 minutes. The fibrin should be collected within an hour. It is collected on glass needles which are made from 3 or 4 mm. Pyrex glass rod or tubing. The tubing is drawn out to form a needle about 3 inches long

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*Parke-Davis & Company, Detroit, Mich.

and 0.3 to 0.5 mm. in diameter. About 3 inches of tubing is left for a handle. The fibrin is collected by slowly lowering the tip of the glass needle into the suspension and rotating it. The collected fibrin is washed by placing the needle in distilled water for a minute or two while the next sample of fibrin is collected and then placing it in ethanol for a like period of time.

If a microbalance is available, the most convenient way to measure the amount of fibrin collected is to dry the needle and fibrin at 80° C. for 30 minutes and weigh it. The weight of the glass needle remains constant, so that only one weighing is required for each determination. The needles are so small that very little time is required for equilibration in the balance. The weighings can be completed in 2 to 4 minutes. The needles are cleaned by dipping them in hot sulfuric acid and rinsing with distilled water.

If a microbalance is not available, the fibrin may be estimated by digesting it in sulfuric acid and hydrogen peroxide, nesslerizing, and determining the color density. The whole operation is conveniently carried out in Pyrex matched colorimeter tubes, which are selected from stock 18 × 150 mm. culture tubes. After the fibrin on the needle tips has been washed with water and alcohol, the needles are inserted in colorimeter tubes which already contain 0.15 ml. of concentrated sulfuric acid. The tubes are then placed in a sand bath on a hot plate, which is adjusted to medium heat so that the acid refluxes. When the tubes have been heated for about 5 minutes, each one is removed from the sand bath and agitated so that the fibrin is brought into contact with the hot acid. The tubes are again heated in the bath and a drop of hydrogen peroxide is added to each. The procedure of heating, agitating, and adding hydrogen peroxide is repeated and the tubes are finally agitated and heated for 5 to 10 minutes in the sand bath. They are then removed and allowed to cool. The sample is now ready for nesslerization. To each tube, 6.0 ml. of water is added and mixed with the acid. Then 4.0 ml. of Nessler's reagent is added and mixed by shaking. The optical density (at 500 mμ) of this mixture is compared with a standard containing 1.0 ml. of water and 4.0 ml. of Nessler's reagent. A reagent blank is used to set the instrument. A Coleman spectrophotometer was used in this work.

Calculations: The gravimetric readings are recorded as milligrams per 0.1 ml. of plasma. These numerical values are also the per cent of fibrinogen in the plasma, so that no conversion is needed.

The colorimetric readings are converted to milligrams of nitrogen by the usual means. Multiplication of this by 6.25 gives milligrams of fibrinogen in good agreement with the gravimetric procedure. The values are recorded as per cent of the plasma actually used. If the blood was diluted with citrate or oxalate anticoagulant solution, the values may be corrected approximately for this dilution. For this purpose it is, of course, necessary to determine the cell volume at the time the blood sample is centrifuged.

TABLE I. PER CENT OF FIBRINOGEN FOUND IN DUPLICATE DETERMINATIONS ON SEVERAL RAT PLASMA SAMPLES

SAMPLE NUMBER		1	2	3	4	5	6	7
DETERMINATION	1	0.301	0.282	0.224	0.202	0.189	0.196	0.186
DETERMINATION	2	0.296	0.280	0.229	0.205	0.195	0.194	0.194

The results presented in Table I indicate the reproducibility of the gravimetric method. The colorimetric method gives the same average value but the range of duplicate determinations is about 0.02 per cent instead of 0.01 per cent for the gravimetric method. In Table II, data are presented on several groups

TABLE II. THE PER CENT OF FIBRINOGEN IN PLASMA FROM HUMANS AND RATS

SOURCE OF PLASMA	NUMBER OF INDIVIDUALS	AVERAGE PER CENT FIBRIN	STANDARD ERROR	RANGE
Normal young men*	6	0.224	0.0220	0.158-0.302
Patients with advanced carcinoma*	22	0.450	0.0316	0.283-0.851
Normal adult carworth-wistar rats†	56	0.222	0.00342	0.153-0.269
Rats given *Serratia marcescens* on day before†	14	0.390	0.0210	0.280-0.524
Adult rats bearing Murphy-Sturm lymphosarcomas†	17	0.414	0.0206	0.278-0.562

*Blood collected in dry oxalated tubes.

†Blood mixed with 10 per cent by volume of citrate. The values reported are not corrected for dilution of the blood by the anticoagulant.

of people and of rats. Human patients and rats with cancer showed the characteristic elevation of the plasma fibrinogen level. Rats given a small dose of sterilized *Serratia marcescens* culture subcutaneously had the characteristic increase in the plasma fibrinogen level the next day. Others have found that the fibrinogen in normal rat plasma averages about 0.25 per cent.[5] When the value in Table II is corrected for dilution, it becomes approximately 0.27. This is satisfactory agreement, since the level varies considerably with the age and diet of the animals. The average value for young men in Table II is lower than the average of 0.27 per cent for 19 normal men reported by Ratnoff and Menzie.[2] However, the two values are not significantly different by "Student's" t test, and the low value reported here is attributed to the youth of the individuals (average age 23) and to the smallness of the sample.

The thrombin level can be reduced from 2.5 to 0.25 units per milliliter without significantly reducing the yield of fibrin in either human or rat plasma. However, since antithrombin activity may be high under some pathologic conditions, the 2.5 unit level is used. The solution may be kept for a week under refrigeration.

Alcohol in the reaction may serve to suppress the plasma antithrombin activity[6] and thus to reduce the chance that the thrombin might be destroyed before clotting is complete. The presence of alcohol greatly facilitates the collection of the fibrin. The clot is stronger and is not attached to the walls of the tube when alcohol is present. This results in a slightly higher and less variable recovery of fibrin compared with that obtained when saline alone is used.

SUMMARY

A new procedure is presented for the determination of fibrinogen in human or rat plasma. The fibrinogen is converted to fibrin by the adding of a solution of commercial thrombin in physiologic saline and alcohol. The fibrin is estimated by drying and weighing it or alternatively by digesting and nesslerizing it directly. The procedure requires 0.1 ml. of normal plasma.

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A METHOD FOR THE SIMULTANEOUS DETERMINATION OF PHENOBARBITAL AND DIPHENYLHYDANTOIN IN BLOOD

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INTRODUCTION

IN THE treatment of chronic diseases such as epilepsy, which require prolonged administration of therapeutic agents, it is desirable to have methods for checking blood levels of the drugs, in order that the adequacy of dosage and possibility of impending toxicity may be appraised. For such purposes, sample size, sensitivity, reproducibility, accuracy, and rapidity of the method are important. In the therapy of epilepsy, both phenobarbital and hydantoins may be administered concurrently. While methods have been described for the determination of phenobarbital and hydantoins when present singly,[1-11] there has been no simple method for their differentiation and determination when both are present in the same sample. In this paper we describe a method for the simultaneous determination of phenobarbital and diphenylhydantoin when both are present in a single blood specimen. This method makes use of the selective extraction of the hydantoin from the blood into an organic solvent, and the return of the two agents to other aqueous phases for individual ultraviolet spectrophotometric quantitation.

A number of authors have reported on the use of ultraviolet spectrophotometry for the determination of barbiturate blood levels.[1-9] Only two methods have been described for hydantoins. Kozelka and Hine[10] determined diphenylhydantoin in biologic material titrimetrically; unfortunately, this method requires large samples and is not rapid. Butler used ultraviolet spectrophotometry to determine plasma levels of 5-ethyl-5-phenyl hydantoin (Nirvanol).[11]

MATERIALS AND METHOD

Reagents.—(1) Sodium bicarbonate, reagent grade. (2) Sodium hydroxide, approximately 10 per cent. (3) Cyclohexane, reagent grade. (4) n-Butanol, reagent grade. (5) Hydrochloric acid, approximately 1 N. (6) Carbonate buffer, pH 11: dissolve 0.1 mole $NaHCO_3$ and 0.09 mole NaOH in 1 liter of distilled water. (7) Acetic acid, approximately 25 per cent v/v. (8) Chloroform, reagent grade. (9) Sulfuric acid, approximately 10 N. (10) Sodium hydroxide, 0.3 N. (11) Saturated potassium bicarbonate: dissolve 50 Gm. $KHCO_3$ in 100 ml. warm distilled water; allow to cool to room temperature and excess $KHCO_3$ to crystallize out; store over excess crystals. (12) Standard solutions of diphenylhydantoin ranging from 5 to 50 mcg. per milliliter: prepare from a stock solution containing 100 mg. of the dried

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acid form dissolved in 100 ml. of carbonate buffer, pH 11. (13) Standard solutions of phenobarbital in concentrations ranging from 5 to 50 mcg. per milliliter: prepare from a stock solution containing 100 mg. of the dried acid form in 100 ml. 0.3 N NaOH. Standard curves are prepared from these standard solutions.

a. *Extraction procedure: Diphenylhydantoin:* Add 5 ml. of oxalated whole blood to a 50-ml. glass-stoppered centrifuge tube containing approximately 0.5 Gm. $NaHCO_3$ and 0.2 ml. 10 per cent NaOH. The resulting pH will be 8.5. Add 20 ml. of cyclohexane and 1 ml. of n-Butanol. Stopper and shake for about five minutes; centrifuge at 2,500 r.p.m. for about five minutes. At this point, the diphenylhydantoin is in the organic phase, and 90 per cent of the phenobarbital remains in the aqueous phase. Transfer the entire cyclohexane phase to another tube containing 10 ml. of 1 N HCl wash (set the tube containing the buffered blood residue aside); stopper and shake this tube for five minutes and centrifuge. At this point, the aqueous phase usually contains a white mass and the cyclohexane phase appears cloudy. Transfer 15 ml. of the cyclohexane phase to another tube containing 5 ml. of carbonate buffer, pH 11, and discard the HCl wash. Shake the tube for five minutes and centrifuge. Aspirate and discard the cyclohexane layer, saving the aqueous phase for quantitation of the hydantoin.

Phenobarbital: Aspirate off any cyclohexane remaining in the tube containing the buffered blood residue saved from the first step. Acidify the blood with 2 ml. 25 per cent acetic acid to adjust the pH to between 5.5 and 6.0. Add 1 ml. at a time, to prevent excessive frothing. When no more CO_2 is being evolved and no more $NaHCO_3$ is visible at the bottom of the tube, add 35 ml. of chloroform. Shake for five minutes and centrifuge. Decant off the aqueous phase above the resulting clotted blood. Break through the blood clot and pour off the chloroform through a Whatman No. 1 filter paper circle into a large glass-stoppered centrifuge tube of no less than 75 ml. capacity. Add 30 ml. of 10 N H_2SO_4 wash; shake for five minutes and centrifuge. Aspirate off the acid phase. Transfer 25 ml. of the chloroform layer to another tube containing 5 ml. 0.3 N NaOH. Shake for five minutes and centrifuge. Centrifugation between 3,000 and 4,000 r.p.m. usually eliminates any turbidity of the final aqueous layer. If an emulsion does form, beating with a glass rod and recentrifuging usually breaks it down. Save the aqueous phase for quantitation of the barbiturate.

Reagent blank: Run 5 ml. of distilled water through the above extraction steps along with the samples. The carbonate buffer from this extraction becomes the blank for the hydantoin; the 0.3 N NaOH becomes the blank for the barbiturate.

b. *Quantitation: Phenobarbital:* Pipette 3 ml. of the 0.3 N NaOH solution into a silica cell and read against its reagent blank, at wave lengths 260, 250, and 240 mμ on the Beckman DU spectrophotometer. Add 0.5 ml. of saturated $KHCO_3$ solution to each cell to bring the pH down to between 10 and 10.5. Again read the samples at 260, 250, and 240 mμ.

Multiply the second readings by 1.17 to correct for dilution, and subtract them from the first readings (0.3 N NaOH). A barbiturate is present if a maximum positive optical density difference occurs at wave length 260, approaching a minimal difference at 250 and a maximum negative difference at 240. Quantitate the amount present by the optical density difference at 260. Determine the amount from the standard curve for phenobarbital in 0.3 N NaOH:

$$10 \text{ mcg./ml.} = 0.205 \text{ optical density units.}$$

Diphenylhydantoin: Pipette 3 ml. of the carbonate buffer (pH 11) into a silica cell and read against its reagent blank at wave lengths 260, 250, 240, and 235 mμ. Add 0.5 ml. 10 per cent NaOH to each cell to bring the pH up to about 12.5 and read the absorption again.

Multiply the second readings by 1.17 to correct for dilution and subtract from them the first readings (pH 11). The optical density differences will show whether a detectable

amount of phenobarbital has passed through the initial extraction step with the hydantoin. If not, the spectrum will not shift and no further correction need be made.

If a detectable amount of phenobarbital has passed through, the spectrum will shift in proportion to the amount present and will exhibit the qualitating characteristics of barbiturates described above. (The amount present may be quantitated by the optical density difference at wave length 260, if desired.) Calculate the absorption due to the phenobarbital in carbonate buffer at pH 11, at 260 and 235 mμ, as follows:

O. D. phenobarbital at 260 = 0.668 × O. D. difference at 260.
O. D. phenobarbital at 235 = 2.12 × O. D. difference at 260.

Subtract these calculated optical densities from those obtained in the first reading (pH 11) to give the true hydantoin readings at wave lengths 260 and 235. This difference bears a direct linear relationship to the amount of hydantoin present. Determine the amount present from the standard curve for diphenylhydantoin in carbonate buffer pH 11:

10 mcg./ml. = 0.196 optical density units.

RESULTS

Table I summarizes the recovery data for known amounts of diphenylhydantoin and phenobarbital when added to normal blood. At levels greater than 9 mcg. per milliliter of the hydantoin, and at all levels of barbiturate, the standard deviation of a single determination is less than ± 8 per cent. Some phenobarbital passes into the cyclohexane phase with the diphenylhydantoin and is quantitated in the carbonate phase. This amount, which averages about 10 per cent (8.6 ± 2.0 to 12.3 ± 1.3 per cent) of the original phenobarbital present, is not included in the table. It is significant only when the total phenobarbital level exceeds 10 mcg. per milliliter (1.0 mg. per cent).

TABLE I. SUMMARY OF RECOVERY DATA ON DIPHENYLHYDANTOIN AND PHENOBARBITAL ADDED TO NORMAL BLOOD

NUMBER OF DETERMINATIONS	MCG. ADDED PER ML.		PER CENT RECOVERY	
	DIPHENYL-HYDANTOIN	PHENOBARBITAL	DIPHENYL-HYDANTOIN*	PHENOBARBITAL†
10	39		79.9 ± 1.9	
10	32		79.0 ± 2.9	
10	38		76.1 ± 2.8	
9	24		81.5 ± 5.2	
10	16		77.3 ± 4.6	
(49)			(78.3 ± 4.4)	
9		36		57.9 ± 3.1
9		23		56.1 ± 3.5
8		19		54.1 ± 5.4
9		14		54.6 ± 1.0
9		6		57.4 ± 2.6
(44)				(56.0 ± 3.4)
6	12	6	82.7 ± 1.2	54.3 ± 2.3
6	9	21	82.8 ± 15.2	61.2 ± 5.3
6	16	19	82.6 ± 7.0	52.1 ± 1.7
6	23	18	81.3 ± 7.8	63.2 ± 7.2
6	26	9	80.7 ± 2.3	58.3 ± 2.4
(30)			(82.0 ± 7.0)	(57.8 ± 6.1)

*Carbonate phase.
†0.3 N NaOH phase only (see text).

Drug-free blood samples from 27 persons and 20 cadavers were analyzed in order to determine blood blanks at the wave lengths utilized. Results are summarized in Table II. For these determinations, the error introduced by substances normally encountered in blood was found to be about 1.5 mcg. per milliliter (0.15 mg. per cent) for diphenylhydantoin and 0.5 mcg. per milliliter (0.05 mg. per cent) for phenobarbital.

TABLE II. OPTICAL DENSITY READINGS OBTAINED ON DRUG-FREE BLOOD AT WAVE LENGTHS CRITICAL TO THE METHOD

EXTRACT	KIND AND NUMBER OF SAMPLES	OPTICAL DENSITY DIFFERENCES			O. D.
		260	250	240	235 MINUS 260
pH 11	Normal 27	+0.002 ± 0.008	+0.006 ± 0.008	+0.007 ± 0.015	+0.020 ± 0.014
	Post mortem 20	+0.004 ± 0.003	+0.001 ± 0.013	+0.008 ± 0.013	+0.033 ± 0.019
0.3 N NaOH	Normal 27	+0.009 ± 0.016	−0.002 ± 0.030	−0.026 ± 0.021	
	Post mortem 20	−0.011 ± 0.018	−0.010 ± 0.007	−0.028 ± 0.018	

Blood samples from ten patients undergoing antiepileptic therapy with diphenylhydantoin sodium (Dilantin), 3-methyl-5-ethyl-5-phenyl hydantoin (Mesantoin), and phenobarbital were analyzed in order to determine the clinical applicability of the method. The results are summarized in Table III.

TABLE III. BLOOD LEVELS OF PATIENTS UNDER DIPHENYLHYDANTOIN, MESANTOIN, AND PHENOBARBITAL THERAPY

PATIENT NO.	DRUG	HYDANTOIN LEVEL MG. PER CENT*	PHENOBARBITAL LEVEL MG. PER CENT*
1	Diphenylhydantoin, mesantoin, and phenobarbital	5.1	2.0
2	Diphenylhydantoin and phenobarbital	1.9	2.0
3	Diphenylhydantoin and phenobarbital	2.3	0.7
4	Diphenylhydantoin and phenobarbital	1.1	0.7
5	Diphenylhydantoin and phenobarbital	3.4	1.4
6	Diphenylhydantoin, mesantoin, and phenobarbital	2.0	0.6
7	Diphenylhydantoin and mesantoin	5.7	--
8	Diphenylhydantoin and mesantoin	2.9	--
9	Diphenylhydantoin	0.9	--
10	Diphenylhydantoin	0.9	--

*Corrected for percentage recovery.

The blood levels were found to run between 0.6 and 2.0 mg. per cent for phenobarbital, and 0.9 and 5.7 mg. per cent for the hydantoin. It will be noted that in those cases where Mesantoin is given together with diphenylhydantoin, a combined hydantoin level is obtained, since it is not possible to differentiate between the demethylated metabolite of Mesantoin and diphenylhydantoin by this method.

DISCUSSION

Cyclohexane itself is a poor solvent for the extraction of diphenylhydantoin. However, the addition of butanol to cyclohexane greatly enhances the percentage of diphenylhydantoin extracted from aqueous solutions. Unfortunately, the addition of butanol also increases the extractability of the phenobarbital in cyclohexane, so that the amount of butanol must be carefully adjusted. The relationship between the amount of butanol added to the cyclohexane and the relative extractability of the diphenylhydantoin and phenobarbital are depicted in Table IV. The higher recovery rates are due to the fact that the solutions

TABLE IV. EFFECT OF n-BUTANOL ON EXTRACTION OF DIPHENYLHYDANTOIN AND PHENOBARBITAL BY CYCLOHEXANE FROM AQUEOUS SOLUTION

RATIO BUTANOL/CYCLOHEXANE	PER CENT RECOVERY DIPHENYLHYDANTOIN	PER CENT PHENOBARBITAL CARRIED OVER INTO pH11 EXTRACT
0/20	4	—
0.5/20	74	12
1.0/20	94	12
2.0/20	95	24
3.0/20	95	34

were acidic and the extractions were carried out from aqueous solutions rather than from blood. These aqueous solutions of the drugs, after acidification, were extracted with a fixed volume of cyclohexane, varying only the amount of n-butanol added. The drugs were quantitated in the carbonate extraction of the organic layer. The ratio of 1 volume of n-butanol to 20 volumes of cyclohexane was found to be optimal for the diphenylhydantoin extraction, while permitting only a small amount of phenobarbital to be simultaneously extracted.

TABLE V. DEPENDENCE OF EXTRACTION ON pH

DRUG	EXTRACTION RATIO*					
	pH 2	6	7	8	9	12.5
Phenobarbital	1.0	1.1	0.68	0.56	0.23	0.23
Diphenylhydantoin	1.0	1.0	1.0	1.0	0.83	0.06

*Assuming amount extracted at most acid pH as the theoretical maximum.

The effect of pH on the extraction of diphenylhydantoin and of phenobarbital was studied, using the 20:1 v/v ratio of cyclohexane and butanol (Table V). When the pH lies between 2 and 6, there is no change in the relative amounts of diphenylhydantoin and phenobarbital extracted by the cyclohexane-butanol system. However, between pH 7 and 9 the quantity of phenobarbital rapidly decreased. Buffering the blood at pH 8.5 was optimal for the extraction of hydantoin, while permitting only a small amount of the barbiturate to be simultaneously extracted. (Buffering with solid sodium bicarbonate and 10 per cent sodium hydroxide was found to be most practical.) Lous[6] reported similar effects of pH on the extractability of phenobarbital from aqueous solutions by chloroform.

The acidification of the blood residue of the first extraction is necessary to bring the pH down to below 7.0, where the partition of the phenobarbital favors the chloroform phase. Two milliliters of any 3 N acid is sufficient. Acetic acid brings the pH to between 5.5 and 6.0. Acidification with the mineral acids, hydrochloric, nitric, or sulfuric, however, brings the pH down to around 2.0, and resulted in a highly colored chloroform layer. Moreover, this pigmentation was carried into the final sodium hydroxide extract, resulting in high blanks.

Because of turbidity, a carbonate buffer was selected as the final aqueous phase for the hydantoin, rather than a sodium hydroxide solution. With sodium hydroxide, turbidity problems were frequently encountered even with an acid wash. Without a preliminary acid wash, even the carbonate buffer would give a turbid solution, but with the acid wash, samples from patients have always been clear, and only the most putrified of the cadaver samples have given turbid extracts.

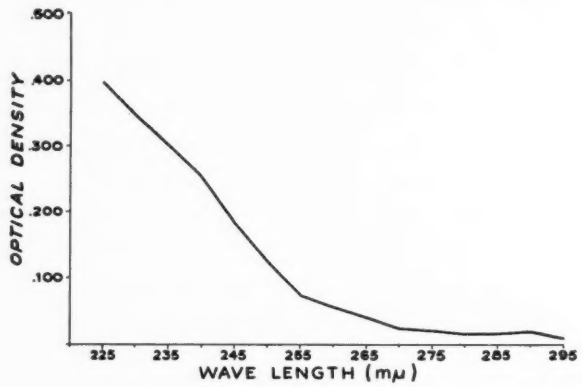

Fig. 1.—Absorption spectrum of diphenylhydantoin in carbonate buffer, pH 11 (12.5 mcg./ml.).

Fig. 1 shows the absorption spectrum of a carbonate buffer solution of diphenylhydantoin at a concentration of 12.5 mcg. per milliliter. The difference between the optical density reading at 235 and that at 260 bears a direct linear relationship to the concentration of the hydantoin, since absorption at both wave lengths follows Beer's law. We found that the spectrum does not shift between pH 10.5 and 12.5, an observation also reported by Goldbaum.[3]

The 5, 5-substituted derivatives of barbituric acid, on the other hand, do exhibit a spectral shift in the alkaline range.[3, 8, 12] One resonating form occurs around pH 10, and another above pH 12. Butler,[8, 12] has attributed this shift to the dissociation of the second proton of the nitrogen. Goldbaum[3] made use of this shift for quantitating and identifying barbiturates. He reported that even in the presence of nonshifting interfering materials which might mask the characteristic spectrum of barbiturates, the qualitating optical density differences already described would indicate the presence of a barbiturate. This observation has been confirmed in this laboratory; we also found that the

relationship holds true between pH 11 and 12.5. Hence in a mixture of phenobarbital and diphenylhydantoin, any shift between pH 11 and 12.5 is due to the barbiturate.

Since the optical densities of the barbiturate and the diphenylhydantoin are additive at the wave lengths utilized, the absorption due to the barbiturate is subtracted from the total reading at pH 11 to give the actual value for diphenylhydantoin absorption. The equations given for the calculation of absorption due to phenobarbital were derived from standard curves at 260 and 235 for phenobarbital in carbonate buffer, pH 11.

The reliability of the correction is clearly illustrated in Table VI. The aqueous solutions A and B were analyzed according to the method. The optical densities are those of the carbonate buffer extract. The sign of the optical density difference at 250 occasionally will be positive instead of negative; however, for this wave length only, the magnitude of the difference, and not its direction, is important. In practice, we have used the direction of the shift at wave lengths 260 and 240 to qualitate the presence of the barbiturate and neglected the direction at 250 as long as the difference remained small. In the data presented in Table I, phenobarbital was detectable in the carbonate fraction at all levels of added drug, down to 10 mcg. per milliliter.

TABLE VI. METHOD OF CORRECTION FOR THE QUANTITY OF PHENOBARBITAL PRESENT IN THE CARBONATE FRACTION WHEN QUANTITATING DIPHENYLHYDANTOIN

	OPTICAL DENSITY READINGS*							O. D. 235-260
	260		250		240		235	
	pH 11	pH 12.5	pH 11	pH 12.5	pH 11	pH 12.5	pH 11	pH 11
Solution A: 50 mcg./ml. diphenylhydantoin	0.132	0.132	0.278	0.282	0.550	0.555	0.657	0.525
Solution B: 50 mcg./ml. diphenylhydantoin plus 58 mcg./ml. phenobarbital	0.204	0.321	0.457	0.463	0.800	0.695	0.898	
O. D. Difference (pH 12.5 minus pH 11)		+0.117		+0.006		−0.105		
Phenobarbital O. D. Correction		−0.078					−0.248	
True O.D. diphenylhydantoin		0.126					0.650	0.524

*Corrected for dilution where necessary.

At levels greater than 10 mcg. per milliliter, 58 per cent of the added phenobarbital was recovered in the sodium hydroxide fraction and 10 per cent in the carbonate buffer fraction, making a total recovery of 68 per cent. Such a recovery compares favorably with the average 70 per cent recovery by other methods devised solely for the determination of barbiturates.[9]

An attempt was made to quantitate the two drugs in a single solution, thereby reducing the number of steps and labor involved in separating them. In pure solutions this was practical. However, in extracting from blood we

were unable to find a common solvent system which would give adequate recovery of both agents while still giving the extremely low drug-free blood blank values obtained in our present method.

Regarding the sensitivity of the method, reference is made to the following facts: If we consider an optical density difference (235 reading minus the 260 reading) of 0.040 units as significant and a recovery of 80 per cent, the blood would have to contain about 0.4 mg. per cent of diphenylhydantoin. Similarly, if we consider an optical density difference at wave length 260 (0.3 N sodium hydroxide reading minus pH 10 reading) of 0.040 units as significant and a recovery of 58 per cent, the blood would have to contain about 0.5 mg. per cent of phenobarbital. The adequacy of this sensitivity for clinical determination is shown in Table III.

For the determination of the relative specificity of the method, the influences of acetylsalicylic acid, trimethadione (3,5,5-trimethyl-2,4-oxazolidinedione), paramethadione (3,5-dimethyl-5-ethyloxazolidine-2,4-dione), Mesantoin (3-methyl-5-ethyl-5-phenyl hydantoin) and Mebaral (N-methylphenobarbital) were studied. When acetylsalicylic acid was added to blood, it was found not to interfere with our determinations. Trimethadione, paramethadione, Mesantoin, and Mebaral were given to dogs in the doses 200, 200, 80, and 50 mg. per kilogram, respectively. Butler had previously shown that at the end of a twenty-four-hour period, both the demethylated and the parent compounds would be present in quantitatable amounts.[8, 11, 13, 14] Blood samples were taken twenty-four hours later, therefore, and subjected to analysis by our standard procedure. Trimethadione and paramethadione did not interfere. However, Nirvanol (5-ethyl-5-phenyl hydantoin), which is the demethylated metabolite of Mesantoin,[11] was detected in the hydantoin fraction; its absorption characteristics are such that it cannot be distinguished from diphenylhydantoin. Mebaral was also found to pass into the cyclohexane phase and appear with the hydantoin. The spectrum of Mebaral, unlike that of phenobarbital, does not shift in the pH ranges utilized here. Consequently, the absorption due to the Mebaral cannot be determined, and corrected for. The demethylated metabolite of Mebaral is phenobarbital[8]; this was quantitated in the sodium hydroxide fraction.

From these experiments, we concluded that among the commonly used antiepileptics only Mesantoin and Mebaral would interfere with the determinations. Whenever levels are determined on patients undergoing antiepileptic therapy, therefore, it is necessary to have a history of medication, insofar as these compounds are concerned, or erroneous interpretation may result.

SUMMARY

A rapid, simple, accurate method of determining diphenylhydantoin and phenobarbital in a single blood sample is described. The separation of the two drugs is based upon differences in partition between blood and a cyclohexane-butanol mixture. After selective extraction of the hydantoin, the two agents are returned to aqueous phases. Final quantitation is carried out by

ultraviolet spectrophotometry. The method is sensitive enough for clinical determinations as well as toxicologic determinations, with a high degree of specificity.

We are grateful to Dr. James M. Fujimoto and Dr. David W. Furnas for their valuable suggestions, and to Dr. Thomas L. Nelson, Sonoma State Hospital, Eldridge, California, for supplying blood samples of patients on anticonvulsant therapy.

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CLEAN PRESURGICAL CLIPPING OF ANIMALS

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PRESURGICAL clipping of small animals for various experimental purposes usually litters the operative skin site, operating table, floor, and general immediate environment with loose hair. This may be prevented by the immediate removal of the hairs as they are clipped by means of a vacuum source which overlies the cutting blade of the clipper. In operation, the cut hair is conveyed under negative pressure through a transport tube to a remote collection bag. In addition to cleanliness, the device aids in "lining up" the hair in front of the cutting blade to provide quicker clipping.

The apparatus used in our laboratory (Figs. 1 and 2) is essentially an animal clipper connected with a remotely placed vacuum cleaner. The vacuum source, above the cutting blades, is a modified gas burner "wing top" (1⅞ inches across wing, ⅝ inch diameter of round end). One of the flat edges of the wing end opening is shortened slightly by grinding on an emery wheel. This is to accommodate a thin, flat holding-plate soldered to the side of the wing top bearing this shortened edge (see Fig. 1). The plate has two notches that coincide with the screws holding the top plate on the cutter head. Thus the screws are used for holding both the top plate of the cutter head and the vacuum source.

Attached to the round end of the wing top is a rubber transport tube (8 feet long, ⅝ inch inside diameter, and ⅛ inch wall thickness), which is clamped to the upper surface of the clipper and taped to the electrical wires leading from the clipper. At the other end of this tube is an adapter for connection with the "attachment" opening of a Eureka vacuum cleaner. To provide as high a vacuum as possible, the floor-sweeping opening of the cleaner is sealed off, and a cheese cloth cover is placed over the dust-removing end of the dust bag. Since ether is used for anesthesia in much of our work, we selected this type of sweeper because the fan compartment is separated from the electric motor. Hence, ether from clipped hair cannot be drawn over the motor as would be the case in many of the "tank type" cleaners.

A single control switch to both vacuum cleaner and clipper is in the 110-volt input line near the clipper. The lead to the vacuum cleaner is tapped off the wire between the switch and clipper. As mentioned above, this lead to the cleaner, the 110-volt input line, and the rubber transport tube are all taped into a common bundle.

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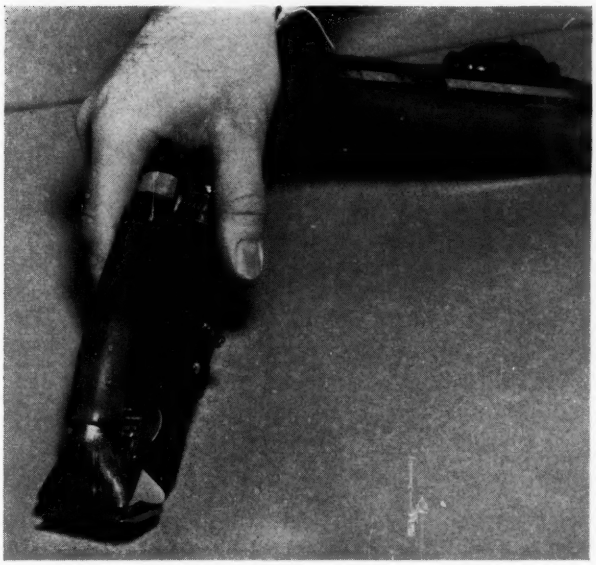

Fig. 1.—Animal clipper showing attachment of overlying vacuum source.

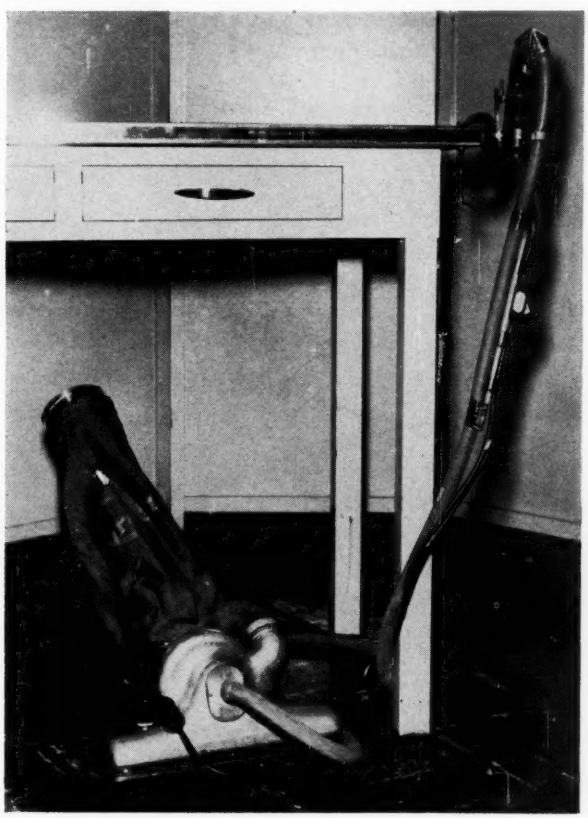

Fig. 2.—Complete unit showing animal clipper and attached vacuum cleaner.

The 8 feet of tubing and cord (which could be extended) between the vacuum cleaner under the worktable and the clipper provide ample freedom of movement for clipping. When not in use, the clipper is suspended from a hook placed on the table's edge, which is made by welding 3/16 inch by 3/16 inch bars to a "C" clamp (see Fig. 2). The clipper has an "eye" on a clamp (modified hose) to fit on this hook.

In Fig. 2, a lightweight plastic bar is inserted between the electrical switch and tubing to prevent breakage in the electrical wiring on either side of the switch.

SUMMARY

An apparatus has been described which provides clean, presurgical clipping of small animals without littering the work area with loose hair. The apparatus is essentially an animal clipper with a vacuum source overlying the cutting blades and connected to a remotely placed vacuum cleaner. Clipped hair is withdrawn immediately through rubber tubing to a remote collection bag.

I wish to thank Mr. H. J. Cromley and Mr. W. H. Cromley for their aid in constructing and perfecting the apparatus.

A DEVICE WHICH ADAPTS A ROLLER TUBE ROTATING MACHINE TO THE ROTATION OF DEEMBRYONATED EGGS

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THE increased use of the deembryonated egg technique of Bernkopf[1] in virus studies[2, 3] has necessitated the use of special equipment for the handling and rotating of these eggs during incubation.

A very simple device which adapts the common laboratory tissue culture roller tube rotating machine to the rotation of deembryonated eggs can be made from a single length of wire.

Enameled wire from a coat hanger serves the purpose well, but stainless steel wire, brass wire, or welding rod of similar gauge is better. The wire is bent freehand with the aid of pliers, or if a large number is to be made, a wooden winding form is suggested. Minor adjustments needed because of variation in egg sizes are easily made by bending the wire slightly. When properly constructed (Fig. 1), the adapter remains open because of the natural

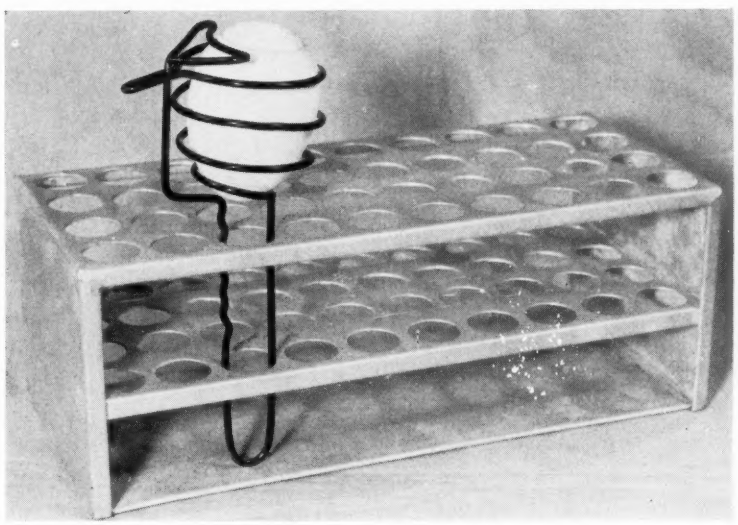

Fig. 1.—Adapter with deembryonated egg, closed upon insertion into test tube rack.

spring of the wire. Compression of the distal portion, which occurs as the adapter is inserted into a hole in a test tube rack or the tissue culture roller tube machine, brings the suitably bent end portion of the wire over the egg,

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effectively locking it into the adapter. Removal of the adapter from the rack or machine automatically releases the egg. A few trials will indicate the proper dimensions of the bends to fit the device to a particular rack or machine.

This adapter eliminates the purchase of a special egg rotator. It fits the ordinary test tube rack so that the device can be used to hold the egg throughout manipulation from deembryonation to incubation.

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DETERMINATION OF PROTEIN-BOUND RADIOIODINE WITH AN ANION EXCHANGE RESIN

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THE standard procedure for determination of protein-bound radioiodine of plasma involves counting of the washed precipitate of plasam proteins. Trichloroacetic acid is the usual precipitating agent used. We found the method inadequate in the presence of a great excess of inorganic radioiodine, such as one observes during the early hours following a dose of I^{131}, being unable to wash away the excess of iodide. An alternative method utilizing an anion exchange resin column was therefore explored, since resins had been used successfully in removing excess radioiodine during the preparation of iodinated serum albumin. Upon making comparisons between results with the resin column and results with trichloroacetic acid precipitation of plasma proteins, under conditions in which both were applicable, we noticed discrepancies, which led to studies of the validity of both methods. The resin column method was found to be both simpler and more valid.

METHODS

Trichloroacetic Acid (TCA) Precipitation.—Five milliliters of plasma was placed in a flat-bottom 20 ml. counting tube, and 10 ml. of 20 per cent trichloroacetic acid was added to precipitate the plasma proteins. After centrifugation, the supernatant was discarded and the precipitate washed four times with 10 ml. aliquots of 20 per cent TCA. Ten per cent TCA gave similar results. Special care was taken not to pour off or remove protein particles in the process of precipitating, stirring, and washing. The packed protein precipitate was then counted in a well-type scintillation counter, previously described.[1] In two instances, the loss of radiothyroxine in supernates was traced by butanol extraction.[1]

Resin Preparation.—Analytic grade Amberlite IRA-400 (OH)* was the resin used. It is necessary to convert the resin from the hydroxyl to the chloride form and to wash and dry it sufficiently so it neither adds nor removes appreciable water from the plasma. Our specific method of preparing the resin follows: (1) A fritted, glass filter funnel, 9.4 cm. diameter, is filled to a depth of 7 to 8 cm. with resin. (2) Successive portions of 1 N HCl are added to cover the resin which is stirred thoroughly. The acid is removed by suction. There is a slight variation initially in the color of different lots

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*Rohm & Haas Co., Philadelphia, Pa.

of resin. After the fourth or fifth wash there is usually a marked color change from amber to tan. One additional wash is used after this color change, although further addition of HCl would do no harm. (3) The resin is washed successively with at least ten aliquots of distilled water. (4) Suction is left on for one hour after the last wash for partial drying (no stirring). (5) The resin is then spread out on an 18 × 20 inch sheet of Whatman 3 mm. filter paper and is mixed with a large spatula for approximately 25 minutes. It is stored at 37° C. in an air-tight jar. (6) The resin is checked for proper moistness by determination of the protein concentration of several plasma samples before and after passage through the resin cascade. We used the biuret procedure.

Resin Procedure.—Pyrex thistle tubes with stems 30 cm. long and inside diameter 3 mm. are constricted about 7 cm. from the tip. A small wisp of cotton is drawn in by suction to the constricted tip. Resin is added to the tube filling the stem to the base of the funnel (approximately 1.5 Gm. of resin in a column 21 cm. long). About 10 ml. plasma is poured into the funnel, passing through the column in approximately 15 minutes. An aliquot of the effluent is counted. Smaller volumes may be used, in which case the proportionate change in plasma water is greater. The resin is discarded after one use. The resin works well with oxalated plasma; however, the effluent from citrated plasma clots or jells.

Dialysis Procedure.—To check the resin's validity in separating inorganic and protein-bound iodine, various plasma samples containing radioactivity were dialyzed. Cellulose Nojax casings,* 24/32, were used. Dialysis was carried out with running tap-water for periods varying from 40 to 77 hours, followed by distilled water for 4 to 6 hours (40 hours in one instance).

RESULTS AND INTERPRETATIONS

Losses Encountered With TCA Precipitation and Washing.—Plasma from four hyperthyroid patients was obtained for study nine or ten days after therapeutic doses (4 to 7 mc.) of radioiodine. At this time nearly all of the radioactivity is present as thyroid hormone and is protein-bound. Counts were made before and after precipitation of proteins and after each of seven washes. The initial radioactivity in the plasma varied from 12,014 to 46,647 counts per minute per milliliter. Table I presents the percentage losses observed with each wash. Typically, approximately 3 per cent of the protein-bound iodine is lost each time TCA is added. The net loss with the standard procedure utilizing four washes in addition to the initial protein precipitation is thus approximately 15 per cent. In the processing of the usual plasma sample twenty-four or more hours after a tracer dose of radioiodine, a minimum of two washes after protein precipitation will generally suffice, though for some plasmas this is not adequate to remove all the inorganic iodide. At very least therefore the loss of protein-bound iodine with the TCA procedure would be 9 per cent.

*Visking Corporation.

TABLE I. PERCENTAGE LOSS OF PROTEIN-BOUND RADIOACTIVITY WITH EACH TCA WASH

WASH	PER CENT LOSS*	CUMULATIVE PER CENT LOSS
1	3.4-5.3	3-5
2	1.5-2.9	5-8
3	2.6-4.2	8-11
4	1.4-3.2	9-14
5	2.1-3.1	12-17
6	1.8-3.0	14-19
7	2.1-3.6	17-23

*Calculated as percentage of the count after TCA precipitation.

TABLE II. PROPORTION OF RADIOACTIVITY LOST IN THE FORM OF HORMONE

	PATIENT TI		PATIENT KA	
	C/M/ML.	PER CENT BEI*	C/M/ML.	PER CENT BEI*
Plasma	33,060	--	59,030	--
Supernates of:				
TCA ppt*	1,609	34	1,752	47
1 and 2 wash	1,374	70	1,660	86
3 and 4 wash	1,069	91	1,447	99

*Abbreviations: BEI, butanol-extractable iodine. Ppt, precipitate.

In two additional instances supernates were extracted with butanol to determine what proportion of the radioactivity washed away was actually thyroxine (Table II). In the initial supernatant, 34 and 47 per cent of the radioactivity was butanol-extractable, and by the third and fourth washes 91 and 99 per cent of the radioactivity was butanol-extractable.

Losses Encountered With Resin Column Method.—Successive passage of plasma through a series of resin columns results in losses similar in magnitude to those with successive TCA washes. Plasmas from two of the posttreatment hyperthyroid patients previously used in the study of TCA washes were passed serially through six resin columns. In the taking of the count after passage through the first resin column as a reference point, the average losses of protein-bound radioactivity in each of the succeeding five resin columns were found to be 3.4 and 5.4 per cent, respectively, for the two plasmas. The plasmas were also passed through serial resin columns, after the adding in vitro of an amount of I^{131} sufficient to double the concentration of radioactivity. The excess radioactivity added was removed with the first resin column after which the serial columns resulted in similar average losses, 3.2 and 4.8 per cent, respectively. Thus about 4 per cent of the protein-bound iodine is lost with passage through one resin column. The procedure recommended for routine use involving but one passage through a single column entails this loss of 4 per cent, in contrast to that of 15 per cent with the TCA method. In addition, the time taken per protein-bound iodine determination with the resin column is about one-fifth that with the TCA method.

Validity of Resin Column Method.—The validity of the procedure recommended was evaluated in two ways: (a) by studying the distribution of I^{131} along the resin column and (b) by contrasting the results with those obtained by dialysis.

TABLE III. DISTRIBUTION OF RADIOACTIVITY ALONG RESIN COLUMN

PLASMAS	1 (C/M)	2 (C/M)	3 (C/M)	4 (C/M)
Total in	12,350	2,670	5,230	27,400
Total out	2,820	1,872	282	623
Retained	9,530	798	4,948	26,777
Per cent removed:				
First cm.	46	66	49	51
Second cm.	26	14	28	30
Third cm.	12	5	12	10
First 3 cm.	84	85	89	91
Last 18 cm.	11	21	5	6
Per cent recovery	95	106	94	97

Plasmas from four patients who had received radioiodine the day before were each passed through a resin column precisely 21 cm. long. The resin columns were cut carefully into 1 or 3 cm. segments, and the radioactivity remaining adsorbed was counted. The results observed are given in Table III. It is clear that most of the iodide is removed in the first 3 cm. Over the last 18 cm., the percentage removed varied from one-third of one per cent to one per cent per centimeter. These results suggest that all the free iodide has been removed since the resin column has much greater capacity than was utilized.

Table IV lists the results of protein-bound radioiodine determinations by both the resin column and dialysis methods. With the use of the dialysis values as reference, the average absolute difference between the methods was 4.5 per cent and the average algebraic difference was –2.4 per cent, with the resin values lower. Conversion ratios based upon the resin method averaged 1.2 per cent lower than those based upon the dialysis method. For practical purposes, the results with the two methods are essentially similar.

TABLE IV. COMPARATIVE RESULTS WITH RESIN COLUMN AND DIALYSIS

PATIENT	TOTAL PLASMA I^{131} (C/M/ML.)	PROTEIN-BOUND I^{131}		CONVERSION RATIO × 100		DIAGNOSIS* AND TYPE DOSE
		RESIN (C/M/ML.)	DIALYSIS (C/M/ML.)	RESIN (PER CENT)	DIALYSIS (PER CENT)	
1	5,355	4,657	4,815	87.0	89.9	Hr, Treatment
2	1,889	48	54	2.5	2.9	Ho, Diagnostic
3	293	19	18	6.5	6.1	Eu, Diagnostic
4	625	154	158	24.6	25.3	Hr(?), Diagnostic
5	138	93	101	67.4	73.2	Hr, Diagnostic
6	15,173	12,925	12,477	85.2	82.2	Hr, Treatment
7	3,156	2,582	2,790	81.8	88.4	Hr, Treatment
8	4,639	1,745	1,739	37.6	37.5	Hr, Treatment
9	3,608	3,348	3,328	92.7	92.2	Hr, Treatment
10	9,017	8,240	7,999	91.3	88.7	Hr, Treatment
11	58,169	3,090	3,255	5.3	5.6	Eu, Treatment
12	12,277	11,351	11,946	92.5	97.4	Hr, Treatment

*Hr, hyperthyroid. Ho, hypothyroid. Eu, euthyroid.

Certain Properties of Resin Removal of Iodide.—The use of resin in a column as herein described was found to be more effective and simple than its use as a mixture with plasma (batchwise use), followed by separation by

centrifugation.[2] Both procedures were carried out simultaneously with six plasma samples to which radioactivity was added. Equivalent amounts of resin were used. The initial range of plasma concentration of radioiodine was 7,010 to 10,426 counts per minute per milliliter. After mixing with the resin batchwise the range of plasma counts was 159 to 477 c/m/ml. After passage through the resin column, the range of plasma counts was 49 to 78 c/m/ml.

The resin column is more effective in removing iodide from plasma than from water. Of 2,300 c/m/ml. added to water and plasma, for example, 365 c/m/ml. remained after passage of the water through one resin column, whereas approximately one-half this amount, 181 c/m/ml., remained after passage of the plasma through a similar resin column. The addition of carrier iodine in nearly optimal amounts (1 mg. = 1/400 of the resin capacity) improves the removal of iodide added to water and plasma, 63 and 38 c/m/ml. remaining, respectively, but the removal from plasma was still more efficient. Carrier iodine does not have this effect on plasma radioiodine appearing in vivo after oral doses. The counts of five plasma samples passed through one resin column without addition of carrier iodine were 121, 62, 125, 37, and 106 c/m/ml. With carrier iodine added, the corresponding counts were 123, 65, 128, 35, and 106 c/m/ml., respectively.

Iodide adsorbed by the resin column cannot be washed off easily. For example two columns retaining approximately 80,000 and 60,000 counts per minute were each washed with 10 ml. of water. Only 15 and 5 counts per minute, respectively, were removed by the water.

Water may be removed from or added to the plasma, depending upon the moisture content of the resin. Care in the preparation of the resin and in its storage will prevent excessive changes in water concentration of the plasma. The changes observed with the procedure described in this report were studied by measuring plasma protein concentration before and after passage through the resin column. Protein concentrations of eleven plasma samples varied from 6.0 to 7.9 Gm. per 100 ml. both before and after passage through one resin column. Individual changes in concentration varied from –0.30 to +0.05 Gm. per 100 ml. The average percentage change was 1 per cent, with a range of 0 to 4 per cent. For 39 additional plasma samples that were passed through two resin columns, the average percentage change in protein concentration was 2 per cent, with a range of 0 to 12 per cent. For practical purposes, the change in water concentration of plasma with passage through one resin column may be ignored. However when the resin is initially prepared for use, changes in plasma protein concentration should be measured as a check on the moisture content of the resin.

SUMMARY

Systematic loss of protein-bound radioiodine has been observed with the standard procedure utilizing protein precipitation and washing with trichloroacetic acid (TCA). Approximately 3 per cent of the protein-bound radioac-

tivity is lost with each wash, with a total loss of about 15 per cent with the usual procedure. By the end of the second TCA wash, over 90 per cent of the radioactivity in the wash solution is butanol-extractable.

Passage of plasma through a column of Amberlite resin removes inorganic iodine efficiently. Protein-bound iodine is also lost, averaging approximately 4 per cent. This is partially compensated for by the loss of a small amount of water by the plasma in passing through the resin column, the protein concentration of the effluent increasing by about 1 to 2 per cent. The first 3 cm. of the 21 cm. resin column remove 80 to 90 per cent of the radioactivity retained by the column, and the last 18 cm. remove 5 to 20 per cent. One passage of plasma through a resin column yields protein-bound radioiodine values that differ by an average of 4 per cent from those obtained with dialysis. Conversion ratios based upon resin values were essentially similar to those obtained with dialysis, being on the average 1 per cent lower.

Passage of plasma through one resin column is recommended as a simple and effective way of removing inorganic I^{131}, no matter how large the amount of radioactivity being processed.

We gratefully record our indebtedness to Mrs. Maud Demorest for her able technical assistance.

REFERENCES

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2. Scott, K. G., and Reilly, W. A.: Use of Anionic Exchange Resin for the Determination of Protein-Bound I^{131} in Human Plasma, Metabolism **3**: 506, 1954.

Erratum

In the article by DR. PHILLIP COMENS entitled "Experimental Hydralazine Disease and Its Similarity to Disseminated Lupus Erythematosus," which appeared in the March issue of the JOURNAL, pages 444-454, Fig. 2 on page 448 is incorrectly labeled. The JOURNAL page should be held in the usual position. The two photomicrographs in the upper half of the figure should be labeled *A* and *B*, respectively. The two lower photomicrographs, from left to right, should be marked *C* and *D*. The legend will then be correct as given.